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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **322nd Meeting** *of the Biochemical Society was held in the Department of Chemical Pathology, Westminster Hospital Medical School, Horseferry Road, London, S.W.* 1, *on Saturday,* 17 *October* 1953, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Phenylalanine and Tyrosine Metabolism in Patients with Phenylketonuria. By R. J. Boscott and H. Bickel.* (*Anatomy Department, The Medical School, Birmingham and The Children's Hospital, Birmingham*)

The urine of patients with phenylketonuria is known to contain an excess of phenylalanine, phenyl pyruvic, phenyl acetic and phenyl lactic acids. Jervis (1953) adduces further evidence that the metabolic error lies in the inability of the liver to convert phenylalanine into tyrosine by *p*-hydroxylation.

We have studied chromatographically (Boscott, 1952*a*, *b*, *c*) the phenolic fractions I, II and III (Boscott, 1952*a*) of the hydrolysed and non-hydrolysed urines from two children with phenylketonuria maintained on (1) a normal diet, (2) a normal diet with supplements of DL-phenylalanine, and (3) a diet free from phenylalanine (described by Bickel, Gerrard & Hickmans, 1953). The urines of twelve more phenylketonuric patients on normal diets were also investigated. All of these subjects excreted in the urine *p*-hydroxyphenyl lactic acid and *p*-hydroxyphenyl acetic acid, both of which are present as abnormal tyrosine metabolites in the urines of scorbutic guinea pigs (Aterman, Boscott & Cooke, 1953). The methods employed do not allow for the detection of the labile *p*-hydroxyphenyl pyruvic acid which also may be present.

Chromatograms of fraction I further revealed the consistent presence of greatly abnormal amounts of compound 30, the chromatographic behaviour and colour reactions of which are identical with those of *o*-hydroxyphenyl acetic acid. The unidentified compound 28, which was always present in normal urines, was not found in the urines of phenylketonurics. Phenyl pyruvic acid was detected on the chromatograms of fraction I as a lemon-yellow azo-derivative, with a bluish halo, by spraying the paper with diazotized ICI 5091 in Na_2CO_3 solution, followed either by heating the paper or by again spraying with 2N aqueous KOH solution.

Fractions II and III consistently revealed large amounts of unidentified fluorescent phenolic compounds, not previously detected in the urines of 140 normal and hospitalized individuals. The urinary excretion of these compounds as well as of *o*-hydroxyphenyl acetic acid and phenyl pyruvic acid was increased after supplements of DL-phenylalanine were added to the diet, but greatly reduced or abolished when the diet contained no phenylalanine. The excretion of *p*-hydroxyphenyl acetic acid and *p*-hydroxyphenyl lactic acid remained virtually unchanged. The excretion of pyrocatechol in fraction II appeared to be less than that normally encountered.

Thus the metabolic error in phenylketonurics would appear to be more complex than has hitherto been recognized. The findings suggest that neither the further hydroxylation of *p*-hydroxyphenyl pyruvic acid nor of *o*-hydroxyphenyl pyruvic acid, to give 2:5-dihydroxyphenyl pyruvic acid with ultimate breakdown of the benzene ring, is possible in phenylketonuric patients. This concept would lend support to the theory of Penrose & Quastel (1937) in addition to that postulated by Jervis (1947, 1950).

It is suggested that *o*-hydroxyphenyl acetic acid and its probable precursors, *o*-hydroxyphenylalanine, *o*-hydroxyphenyl pyruvic acid and *o*-hydroxyphenylethylamine, should be examined as possible causal agents of the amentia present in phenylketonurics.

* In receipt of a grant from the Medical Research Council.

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Alkyl Sulphides Evolved from Dog's Urine by Sodium Hydroxide. By F. CHALLENGER, D. LEAVER and MARGARET I. WHITAKER. (*Organic Chemistry Department, University of Leeds*)

Abel (1895) concluded on slender evidence that the compound evolved from dog's urine by boiling alkali was diethyl sulphide. Neuberg & Grosser (1905–6) claimed, without details, to have obtained the phosphotungstate and bismutho-iodide of $[Me.\overset{+}{S}.Et_2]\ \bar{O}H$ from the urine. M. I. Whitaker converted the evolved sulphide R.S.R′ into the mercurichloride, mercuribromide, benzylsulphonium picrate and styphnate. Their melting points differed from those of corresponding diethyl sulphide derivatives, approximating to those of methyl-*n*-propyl sulphide. The derivatives were non-homogeneous. Mercurichloride analysis suggested

$$2CH_3.S.C_3H_7.5HgCl_2.$$

The sulphilimine, $R.R'.S \rightarrow N.SO_2.C_6H_4.Me$, on repeated crystallization had m.p. 103–104° and 104–105° mixed with methyl-*n*-propylsulphilimine, m.p. 105°.

The sulphide from dog's urine was converted to the methylsulphonium base (X) $[R.\overset{+}{S}.R'(Me)]\ \bar{O}H$. This was chromatographed (acid-washed paper, butanol-CH_3COOH; bromophenol blue) simultaneously with mixtures of (*a*) $[Me_2\overset{+}{S}.n\text{-}Pr]\ \bar{O}H$ with traces of (*b*) $[Me_2\overset{+}{S}.n\text{-}Bu]\ \bar{O}H$ and (*c*) $[Me_2.\overset{+}{S}.iso\text{-}Bu]\ \bar{O}H$, respectively. Pattern (*a* + *b*) was identical with X, pattern (*a* + *c*) was different.

The sulphilimine from dog's urine was then chromatographed (Perspex-coated paper, ethyl acetate; aqueous KI + HCl; iodine stain). Two spots appeared. Corresponding sections were removed, separately boiled with $NaHSO_3$ and the evolved sulphides converted into mercurichlorides. The main band gave m.p. 164–165° and mixed m.p. 165° with $2Me.S.n\text{-}Pr.5HgCl_2$, m.p. 165°. The minor band, m.p. 110–114°, sintering slightly at 90°, behaviour identical with that of Me.S.*n*-Bu mercurichloride.

The sulphide from dog's urine is therefore mainly methyl-*n*-propyl sulphide with some *n*-butylmethyl sulphide.

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Enzymic Synthesis of Alkyl Esters of Ribomononucleotides. By L. A. HEPPEL and P. R. WHITFELD (introduced by R. MARKHAM). (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

Various alkyl esters of ribomononucleotides have been synthesized chemically, and it has been found that some of them are hydrolysed by pancreatic ribonuclease (Brown & Todd, 1953). We have now been able to demonstrate their enzymic synthesis by ribonuclease and by a fraction from spleen.

Ribonuclease catalyses the synthesis of cytidine methyl (or ethyl) phosphate from cytidine-2′:3′ phosphate and the appropriate alcohol. The identification of the product is based upon its R_F values in two solvents and ionophoretic mobility at pH 3·5, compared with the chemically synthesized ester (from Dr D. M. Brown). Further, its stability to 0·1 N-HCl corresponds to that of alkyl esters, in contrast to cytidine-2′:3′ phosphate which is more acid labile. The enzymic product is split during prolonged digestion with ribonuclease to give cytidylic acid. Uridine-2′:3′ phosphate participates in similar reactions.

Ribonuclease also catalyses the formation of cytidine methyl phosphate from cytidine-3′ benzyl phosphate and methanol. Cytidine-2′ benzyl phosphate is inactive, a result to be expected, since Brown & Todd (1953) found that only the 3′-ester is hydrolysed by ribonuclease. Paper chromatography of the product in ammonium sulphate–sodium acetate–*iso*propanol (Markham & Smith, 1952) shows only one spot, running in the position of one of the chemically synthesized isomers, and presumably is cytidine-3′ methyl phosphate.

Experiments with an enzyme fraction from spleen (cf. Heppel, Markham & Hilmoe, 1953) have also shown the formation of cytidine methyl phosphate from cytidine-3′ benzyl phosphate and methanol. In contrast to ribonuclease, however, spleen enzyme catalyses, in addition, the production of methyl and ethyl esters of adenylic acid from the corresponding benzyl ester.

In the above reactions one can demonstrate the nearly complete disappearance of the cyclic mononucleotide or of the benzyl ester and a substantial accumulation of the methyl ester with 1 M methanol and the other reactants present in a concentration of 0·01 M. The reactions proceed to a considerable extent with 0·25 M methanol.

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Exchange Reactions Involving Dinucleotides and Cyclic Mononucleotides. By L. A. Heppel, P. R. Whitfeld and R. Markham. (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

Pancreatic ribonuclease has been found to catalyse the formation of a dinucleoside monophosphate from a cyclic pyrimidine mononucleotide and a pyrimidine nucleoside. Two reactions of this type have been demonstrated:

(1) Dicytidine monophosphate is formed from cytidine-2′:3′ phosphate and cytidine.

(2) Uridylyl cytidine is formed from uridine-2′:3′ phosphate and cytidine.

With excess nucleoside a large fraction of the cyclic mononucleotide reacts to form the dinucleoside monophosphate. The identification of the product is based upon its R_F value in *iso*propanol-water-NH_3 and in ammonium sulphate-sodium acetate-*iso*propanol (Markham & Smith, 1952), a base:phosphorus ratio of 2:1, and the fact that further digestion of the product with ribonuclease yields equal quantities of mononucleotide and nucleoside. In the case of dicytidine monophosphate, the ionophoretic mobility of the enzymatic product at pH 3·5 is 0, whereas the starting materials have a considerable mobility in opposite directions. There is evidence that carbon 5′ of the nucleoside is esterified in forming the new diester link because the product is oxidized by periodate, and is also hydrolysed to nucleosides by snake venom. The enzymic product is also attacked by ribonuclease, and therefore carbon 3′ of the cyclic mononucleotide is involved in forming the phosphate bridge between the nucleoside residues.

With an enzyme fraction from spleen (cf. Heppel, Markham & Hilmoe, 1953) several exchange reactions have been demonstrated:

(1) Adenylyl uridylic acid + cytidine → adenylyl cytidine + uridylic acid.

(2) Adenylyl uridylic acid + methanol → adenosine methyl phosphate + uridylic acid.

Both of these reactions proceed to a considerable extent as written; their reversibility has not been investigated.

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The Dephosphorylation of Thiamine Pyrophosphate by Suspensions of Cerebral Tissues. By O. E. Pratt. (*Departments of Biochemistry and Neuropathology, Institute of Psychiatry, British Post-graduate Medical Federation, Maudsley Hospital, London, S.E.* 5)

Thiamine pyrophosphate is hydrolysed by brain and by other animal tissues (Peters, 1937; Ochoa, 1939; Westenbrink, Steyn Parvé & Goudsmit, 1943). Histochemical investigations into the localization of enzymes in brain showed that the histological sites of thiamine pyrophosphate hydrolysis did not correspond with the sites of glycerophosphate hydrolysis by the non-specific acid or alkaline phosphatases (Naidoo & Pratt, 1952, 1953). Confirmation of the presence of a separate enzyme has been provided by the following study.

Determinations were made of the rates of inorganic phosphate liberation, by fresh saline suspensions of whole rat brain, from glycerophosphate at pH 5·3 (acetate buffer) or pH 9·1 (veronal buffer), or from thiamine pyrophosphate at pH 6·9 (maleate buffer). The rate of thiamine pyrophosphate hydrolysis was maximal at pH 6·9, falling off more rapidly on the acid side than on the alkaline, whilst that of glycerophosphate was minimal at about pH 6·9. The optimal thiamine pyrophosphate concentration was 10^{-3} M. Considerable activation was produced by Mg^{2+} (0·03 M) or Ca^{2+} (0·05 M), the latter being used routinely. The acid phosphatase hydrolysing glycerophosphate differed in that a high proportion of the activity was obtained in the absence of added divalent cations.

There were differences in the inhibitor concentrations affecting the different reactions. Thus, the ratio $(Inh.)_{50}$ thiamine pyrophosphatase/$(Inh.)_{50}$ acid

phosphatase was 190 for NaF but only 0·1 for $La(NO_3)_3$. The ratio $(I)_{50}$ thiamine pyrophosphatase/$(I)_{50}$ alkaline phosphatase was 200 for cysteine. An acetone dried brain suspension showed almost no thiamine pyrophosphatase activity, although retaining the ability to hydrolyse glycerophosphate. A brain suspension prepared by grinding in veronal buffer (pH 9·1, 0·1 M) showed high thiamine pyrophosphatase activity but much reduced non-specific acid phosphatase activity. Confirmation that thiamine pyrophosphate is hydrolysed by an enzyme that is distinct from the alkaline phosphatase is provided by the histological localization of the former mainly in nerve cell bodies and the latter mainly in blood capillaries. The differential effects of inhibitors or of acetone or alkali treatment also distinguish the thiamine pyrophosphatase from the acid phosphatase hydrolysing glycerophosphate.

These findings indicate that the dephosphorylation of thiamine pyrophosphate by brain is carried out by a separate enzyme.

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The Distribution of Radioactivity in Goat Casein after Injection of Radioactive Amino Acids and its bearing on Theories of Protein Synthesis. By B. A. Askonas, P. N. Campbell and T. S. Work. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Four radioactive amino acids, [^{14}C]glycine, [^{14}C]-valine, [^{14}C]lysine, and [^{35}S]methionine, were injected simultaneously into a lactating goat and milk samples were collected at 4 and 8 hr. A 1 g. sample of the 4 hr. casein was hydrolysed for 18 hr. at 105° with 6 N-HCl and a second sample for 12 hr. at 56° with conc. HCl. Five radioactive amino acids were isolated from the complete hydrolysate and had the following relative specific activities (counts/min./cm.^{2}): glycine 11 400, serine 1510, valine 1008, lysine 3170, methionine 625. The partial hydrolysate was fractionated first on charcoal, then on buffered Dowex 50, and finally, after conversion of the peptides into dinitrophenyl-peptides, on buffered silica columns. The following peptides were isolated: leuc.gly., thre.[gly.glu.], thre.[asp.asp.val.], val.-[pro.leuc.val.], val.[?] (accidental loss of water-soluble fraction of DNP-peptide hydrolysate), lys.[asp.glu.leuc.phe.], lys.[glu.phe.lys.], ser.pro. and ser.leuc. Identification was provisional and depended on paper chromatography; the compound listed as a lysine pentapeptide may be a mixture of two lysine tripeptides. The sequence of amino acids enclosed by square brackets was not determined. Each peptide was hydrolysed, the radioactive amino acid isolated as the dinitrophenylamino/acid, and its relative specific activity determined. The four radioactive valines gave counts of 1120, 1040, 1680 and 1100; the three lysines gave counts of 2780, 3040 and 2950: two serines had counts of 1475 and 1334, and two glycines counts of 11 200 and 10 200. The amino acids isolated from different peptide sequences must come from different parts of the casein molecule but, except for one valine, all amino acids isolated from peptides had substantially the same radioactivity as the corresponding amino acid isolated from a complete hydrolysate of casein.

These results suggest that casein is synthesized from free amino acids and that no peptides are supplied for casein synthesis by partial breakdown of plasma protein. They do not exclude the possibility that peptide intermediates are formed during protein synthesis, but they indicate that any such peptide must have a transient existence.

Some Effects of Inorganic Phosphate and Bicarbonate on Cell Survival and Proliferation in Chemically Defined Nutrient Media. By Charity Waymouth. (*Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A.*)

The common physiological salt solutions used as components of tissue culture media (Tyrode, 1910; Pannett & Compton, 1924; Gey & Gey, 1936; Earle, 1943; Hanks, 1948) contain amounts of inorganic phosphate of the order 0·4–1·0 mM. Simms & Sanders' (1942) solution X 6 for adult fibroblasts contains 1·5 mM, and the Z 16 solution designed for adult epithelium contains 9·0–12·0 mM phosphate (Parshley & Simms, 1950). Pannett & Compton's (1924) solution, and the high phosphate Z 16 solution of Parshley & Simms, contain no bicarbonate. The other salt solutions contain

bicarbonate at (Earle, 1943, 26·0 mM) physiological levels or lower (e.g. Tyrode, 1910, 12 mM; Hanks, 1948, 1·5 or 4·2 mM), and either monobasic or dibasic phosphate or both in a buffer of pH 7·4–7·8.

The synthetic nutrient solutions hitherto described (White, 1946; 1949; Morgan, Morton & Parker, 1950) have contained amounts of phosphate and bicarbonate based on the concentrations in balanced salt solutions designed to imitate blood plasma. The classical biological nutrient media contain extracts of embryonic tissue in addition to plasma or serum. The total phosphorus of chick-embryo extract (1:1) as usually prepared is 8·0–10·0 mM (Waymouth & Laurence, unpublished), or several times the amount provided in plasma or the usual salt solutions. It has been shown (Harris, 1952) that, in the presence of dialysed embryo extract, chick-embryo tissues exhibit a specific requirement for bicarbonate at about 25 mM, that 1·0 mM inorganic phosphate is adequate for growth in the presence of available organic phosphate and that 3·0 mM supplementary inorganic phosphate is inhibitory.

In the present study of the survival, maintenance of function and proliferative activity of cells in simple, chemically defined media, it has been found that these properties of the cells are all enhanced by increasing the amounts of inorganic phosphate in the synthetic media to about 2 mM. Inorganic P has proved to be a limiting factor for survival, for maintenance of heart-muscle contraction and of ciliary activity in lung epithelium. This suggests that one of the so-called 'growth factors' in embryo extracts is inorganic phosphate, or labile phosphate readily available. Besides contributing to the buffering capacity of the medium, the phosphate probably exercises an important function in maintaining the appropriate conditions for phosphorylation and the production of high-energy phosphate. In the presence of 26·6 mM glucose (480 mg./100 ml.) optimum concentrations of inorganic P and bicarbonate for several types of cell have been found to fall in the range 1·77–2·36 mM-P and 19·95–26·6 mM-$NaHCO_3$.

This work was done during the tenure of an American Cancer Society British-American Exchange Fellowship, recommended by the British Empire Cancer Campaign and the Committee on Growth of the National Research Council of the U.S.A. The author is grateful for the opportunities afforded by this Fellowship; to Prof. A. Haddow and the Board of the Royal Cancer Hospital, London, for leave of absence; and to Dr P. R. White for constant encouragement.

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Examination of the Effect of *Clostridium oedematiens* Toxin on Certain Enzyme Systems.

By Gillian M. Lewis and Marjorie G. Macfarlane. (*Lister Institute, London*)

Culture filtrates of *Clostridium oedematiens* injected into animals produce a gelatinous oedema and marked haemo-concentration. This suggested that the lethal factor, the α-toxin, might inhibit some enzyme system maintaining selective permeability. The action of the toxin *in vitro* on a number of respiratory and other enzymes was therefore examined. In complex systems, the effect of the toxin on each component (apo-enzyme, coenzyme, substrate) was examined in turn by making the particular component the rate-limiting factor in the reaction.

The toxin used, derived from *Cl. oedematiens*, type B, strain Albiston, contained 4000 (mouse) LD_{50}/mg. N; it had slight lecithinase activity, approx. equivalent to 0·24 LD_{50}/mg. N, but no other haemolytic activity. The enzyme or other component examined was incubated in small quantities with and without 500–1000 LD_{50} toxin at about neutral pH for 10–20 min. at 38°; the remaining components were then added and the relative enzymic activities with and without toxin compared.

No effect of the toxin was observed on any component of the following systems: (muscle) glycolysis of glycogen and glucose; Lohmann's enzyme, creatine, phosphocreatine; diphosphopyridine nucleotide-dependent dehydrogenases; (kidney) D-amino acid oxidase; (liver) acetylating enzyme and coenzyme A; catalase; (blood) acetylcholine esterases, carbonic anhydrase; (yeast) hexokinase, carboxylase; (milk) xanthine oxidase.

The oxygen uptake of a rabbit-muscle brei was inhibited 50% after preincubation with toxin (1000 LD_{50}/mg. fresh tissue); the cytochrome oxidase activity of rat-liver mitochondria was also reduced by the toxin in absence of added cytochrome, but not if cytochrome *c* were added. This inhibition, however, was not due to the lethal component (α-toxin) of the crude toxin, as it was not prevented by pre-neutralization with the specific antitoxin. The lecithinase activity of the toxin was insufficient to account for this effect. Oxidative phosphorylation by rat mitochondria in presence of L-glutamate, adenylic acid and added cytochrome *c* was not affected by the toxin.

The possibility that the lethal toxin is present in the culture filtrates as a 'protoxin', activated *in vivo* by tissue constituents, was offset to some extent by the use of crude tissue enzyme preparations; addition of boiled tissue extracts or cysteine had also no activating effect. Though the possibility of false negative results cannot be completely excluded it seems unlikely that the *Cl. oedematiens* lethal toxin directly affects the energy-producing systems of tissues.

Effects of Adrenal Corticoids on Lipogenesis in Mammary Tissue *in vitro*. By JUDITH H. BALMAIN, S. J. FOLLEY, R. F. GLASCOCK and MARY L. MCNAUGHT. (*National Institute for Research in Dairying, University of Reading*)

We have shown that cortisone, *in vitro* at high concentrations, inhibits lipogenesis by mammary-gland slices from lactating rats and partially blocks the stimulatory effect of insulin on this process (Balmain, Folley & Glascock, 1952). The effects of adrenal corticoids on the incorporation of glucose and acetate carbon into fatty acids by lactating mammary slices from the rat and ewe have been further studied. In some cases ^{14}C and ^{3}H were assayed by gas counting (Glascock, 1952); in others ^{14}C only has so far been assayed by end-window counting.

In rat slices (substrate: [^{14}C]glucose + [Me-^{3}H]-acetate) cortisone (approx. 100 μg./ml.) decreased the incorporation of acetate and glucose carbon, confirming our previous finding. Incorporation of glucose carbon was also strongly inhibited by deoxycorticosterone (100 μg./ml. but not 50 μg./ml.) and corticosterone (100 μg./ml.). On the other hand, 17-hydroxycorticosterone (100 μg./ml.) had no inhibitory effect on the incorporation of glucose or acetate carbon and did not antagonize the effect of insulin. The results for sheep slices were somewhat different. Cortisone (100 μg./ml.) had no consistent effect either way on the incorporation of acetate or glucose carbon (substrate: [^{14}C]glucose + [Me-^{3}H]acetate in some experiments; [1-^{14}C]acetate + glucose in others); nor had 17-hydroxycorticosterone (100 μg./ml.) any consistent effect on acetate carbon uptake (substrate: [1-^{14}C]acetate + glucose). Deoxycorticosterone and corticosterone (100 μg./ml. but not 50 μg./ml.) both strongly inhibited the uptake of acetate carbon (substrate: [1-^{14}C]acetate + glucose).

The absence of an inhibitory effect of 17-hydroxycorticosterone on lipogenesis in rat mammary slices by contrast with the marked inhibitory effect of equal concentrations of all other corticoids studied, whether oxygenated at $C_{(11)}$ or not, is of interest in view of the possibility that 17-hydroxycorticosterone may be the natural hormone of the adrenal cortex. This may not be so in the rat, for Bush (1953) reports that rat adrenal vein blood contains corticosterone and 17-hydroxycorticosterone with the former greatly predominating. In the rat some control of lipogenesis could perhaps be exercised through changes in the proportion of these two steroids secreted by the adrenals. The different response of sheep udder tissue to cortisone is of interest in connexion with previously observed metabolic differences *in vitro* between mammary tissue from ruminants and non-ruminants (Balmain, Folley & Glascock, 1953).

We are indebted to Dr C. C. Porter, of the Merck Institute for Therapeutic Research, for cortisone (free alcohol), to Dr A. White, formerly of Chemical Specialities Co. Inc., for corticosterone and 17-hydroxycorticosterone, and to Dr W. J. Tindall of Organon Laboratories Ltd. for deoxycorticosterone.

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The Effect of an Anti-thyroxine Compound on the Deiodination of Triiodothyronine in Rats. By J. H. Wilkinson and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, W.S.* 1)

In an attempt to explain the metabolic effects of the anti-thyroxine compound, butyl 4-hydroxy-3:5-diiodobenzoate (BHDB), in mice, Maclagan, Sprott & Wilkinson (1952) suggested that BHDB might act by inhibiting deiodination processes. If in the ordinary course of metabolism, thyroxine undergoes conversion into triiodothyronine and the latter is further deiodinated to inactive substances, then interference with these processes would lead to diminution of the effects of thyroxine and enhancement of those of triiodothyronine, as actually observed.

The first part of this hypothesis was tested and BHDB was shown to reduce the extent of iodide excretion in the urine of rats after treatment with thyroxine (Wilkinson & Maclagan, 1953). It remains to ascertain whether the drug has a similar effect when triiodothyronine is given.

Triiodothyronine labelled in the 3′-position with ^{131}I was given subcutaneously in a dosage of 6 μg./rat to four groups of three rats (body wt. 140 ± 15 g.). Two of the groups were also given 50 mg./rat BHDB orally on the previous day, and 25 mg./rat daily throughout the period of the experiment. The remaining two groups received the medium (1% methoxycarbonylcellulose) in place of BHDB. Each group was kept in a metabolism cage and the urine and faeces were collected daily. The urinary and faecal radioactivity were measured in a Veall liquid counter. A similar experiment was performed in which triiodothyronine was given at a dosage of 30 μg./rat.

The percentages of the dose excreted in the urine during 5 days by the eight groups of rats were: triiodothyronine (6 μg./rat), 25·9, 25·0; triiodothyronine (6 μg./rat) + BHDB (50 and 25 mg./rat daily), 8·5, 13·4; triiodothyronine (30 μg./rat), 20·6, 20·1; triiodothyronine (30 μg./rat) + BHDB (50 and 25 mg./rat daily), 9·0, 10·5.

The combined urinary and faecal excretion of all groups over the 5-day period was 65–80% of the injected dose.

Since the urinary radioactivity has been shown to consist of iodide and not thyronine derivatives (Wilkinson & Maclagan, 1953), it is clear that BHDB exercises a marked restraint on the deiodination of triiodothyronine. The results therefore support our hypothesis as to the mode of action of BHDB.

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The Detection of Thyroxine and Related Substances on Paper Chromatograms by the Ceric Sulphate-Arsenious Acid Reaction. By C. H. Bowden and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School,* 17 *Horseferry Road, London, S.W.* 1)

In the course of work on the urinary excretion of anti-thyroxine compounds (Maclagan & Wilkinson, 1951; Fraser & Maclagan, 1953), it was noted that the ceric sulphate-arsenious acid test for iodides was also applicable to certain iodine-containing organic compounds. The application of this method to the demonstration of thyroxine and related substances on paper chromatograms was therefore studied. Preliminary attempts in which the reagent was sprayed on the paper were unsuccessful, but after many trials satisfactory results were obtained by saturating a second blank paper with the reagent and then applying the chromatogram to this between glass slides. The detailed technique was as follows:

Equal volumes of 10% (w/v) $Ce(SO_4)_2.4H_2O$ in N-H_2SO_4 and 5% (w/v) sodium arsenite in N-H_2SO_4 are mixed and evenly applied to a no. 1 Whatman chromatographic paper strip of the same size as the chromatogram, by laying the paper flat on a sloping clean glass plate and allowing the reagent to soak the paper. The dry chromatogram is then placed on the wet sheet and another glass plate placed on top and pressed firmly down. After development for 30 min. both papers are dried in an iodine-free atmosphere.

The iodine-containing compounds are indicated by white spots on a yellow background. The chromatogram when dry is only stable for 1–2 days, but permanent records can be made either by direct photography with a blue filter or by backing the chromatogram with photographic paper followed by exposure to ultraviolet light. Paper stained with ceric sulphate interrupts the transmission to ultra-

violet light, whereas the white spots do not. Alternatively, the paper may be interposed between the ultraviolet lamp and a photoelectric cell, as described by Nanninga & Bink (1951) in a different connexion, and the resulting current measured. In this method the lead-glass screen recommended by these workers is omitted.

The limiting quantities detectable with typical compounds by the various methods of recording after chromatography for 16 hr. in a butanol-dioxan-ammonia system at room temperature were determined. It was found that the sensitivity is many times greater than that of previous methods for these compounds. By the photoelectric method semi-quantitative results can be obtained in the range from 0·01 to 0·1 μg. for KI and from 0·1 to 1 μg. for thyroxine or triiodothyronine. The method is applicable to a wide range of organic and inorganic iodine compounds.

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Fractionation of the Intracellular Material of *Bacillus anthracis* Grown *in vivo*; the Polysaccharide and Polyglutamic Acid. By H. Smith and H. T. Zwartouw. (*Microbiological Research Department, Porton, Wilts*)

Bacillus anthracis isolated from infected guinea pigs, and differing from organisms cultured *in vitro*, lysed in $(NH_4)_2CO_3$ solution (0·16 % w/v) at 0°. The solution (pH 8·5) lost all $(NH_4)_2CO_3$ on freeze-drying (Smith, Keppie & Stanley, 1953*a*, *b*).

Dialysis removed 50 % of the material, including solids from the Locke's solution in the original bacterial sludge (Smith *et al.* 1953*b*). The dialysate (1·6 % w/v) was fractionated at pH 7. The fractions and their essential nature were as follows: (1) barium acetate (1 % w/v) added at 20° (yield 7 %, protein); (2) ethanol added to 5 % v/v at 0° (yield 8 %, polyglutamic acid); (3) ethanol added to 33 % v/v at 0° (yield 14 %, protein); (4) material remaining soluble (yield 9 %, polysaccharide). Each fraction was treated with Na_2SO_4 to precipitate barium, centrifuged, dialysed and freeze-dried.

The polysaccharide (1 % w/v) in sodium acetate solution (1 % w/v) was fractionated with ethanol (50–70 % v/v). Fuller's earth powder removed small amounts of bacterial protein. The sample was ultracentrifugally and electrophoretically homogeneous at pH's 4 and 8 and modified partial solubility studies gave no evidence for heterogeneity. It had galactose (anthrone method), 43·0 % (± 2); hexosamine, 37·0 % (± 2); N, 3·95; distillable acid after hydrolysis calculated as acetyl, 14·7 %; $[\alpha]_D^{20} + 81° \pm 5°$. Paper chromatography showed galactose, glucosamine, and ninhydrin positive spots different from those due to glucosamine and equivalent to the amino acids in 4 % of bacterial protein. Repeated treatment with fuller's earth, which removed bacterial protein completely from solution, has not reduced the N or amino acid figure. This analysis is different from that of a polysaccharide extracted by more drastic methods from *B. anthracis* cultured *in vitro* (Ivanovics, 1940). A dilution (1–640000) of the polysaccharide precipitated serum provided by the Instituto Seroterapico Milanese Serafino Balfanti for the Ascoli thermo-precipitation test.

Polyglutamic acid was purified by refractionating with barium acetate and ethanol and treatment with fuller's earth powder. It was electrophoretically and ultracentrifugally homogeneous at pH's 4 and 8, and had N, 9·0; sulphated ash, 44·5; foreign amino acids, nil (less than those in 0·2 % of bacterial or plasma protein); carbohydrate, nil; $(C_5H_6O_3NNa)_n$ requires N, 9·3; sulphated ash, 47·0 %.

Some properties of the protein fractions are described in the following abstract.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to make this communication.

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The Nature of the Aggressins Produced by *Bacillus anthracis* Growing *in vivo*. By H. Smith, H. T. Zwartouw and R. C. Gallop. (*Microbiological Research Department, Porton, Wilts*)

Keppie, Smith & Harris-Smith (1953) have shown that *Bacillus anthracis* when growing in guinea pigs produces aggressins intracellularly, and also extracellularly in the body fluids of the host. Both products have now been fractionated. Aggressin activity has been measured by the capacity to interfere with the phagocytosis of virulent *B. anthracis* (grown in broth) by guinea-pig phagocytes (Keppie *et al.* 1953). The concentrations (w/v) of fractions used in this test are given in brackets.

Of the intracellular products (see previous abstract) diffusate (2·5%) and the polysaccharide (2·5%) were inactive, while polyglutamic acid was active at high concentrations (2%). High activity was found in the protein fractions (0·025%), which after refractionation with barium acetate and ethanol were free from polyglutamic acid and the polysaccharide.

Extracellular products were separated efficiently from plasma protein in the body fluids by barium acetate/ethanol fractionation. Freeze-dried material in water (8% w/v) at pH 7 and 0°, was dialysed (diffusate, yield 50%, inactive (1·6%)) and treated with barium acetate (2% w/v). Fractions were taken at the following ethanol concentrations, and recovered as described in the previous abstract. (i) 5% ethanol, yield 1%, containing 60–70% polyglutamic acid. When purified as described for the polyglutamic acid in the previous abstract it had the same criteria of purity and was active at high concentrations (2%). (ii) 10% ethanol, yield 1%. After refractionation it had N, 13·2%; ash, 2·8%; all the usual amino acids; polyglutamic acid, nil; nucleic acid, nil; carbohydrate (Molisch), 1·5%; hexosamine, 1·5%; and was highly active (0·01%). (iii) 20% ethanol, yield 3%, small activity (0·2%). (iv) Soluble material, yield 35%, inactive (3·2%).

Polyglutamic acid, concentrated in the capsule surrounding *B. anthracis* may help to prevent phagocytosis, but the most powerful aggressin resides in highly active, essentially protein fractions isolated from inside and outside the cell. The latter have received little attention in previous chemical studies on *B. anthracis*.

Parallel tests for protective antigen (Keppie *et al.* 1953) on the fractions from the body fluids showed diffusate inactive (12·8%), polyglutamic acid inactive (0·8%), 10% fraction highly active (0·025%), 20% fraction slightly active (0·2%), and soluble material inactive (3·2%). Concentration of aggressive and protective activity in the same fraction is noteworthy.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to make this communication.

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Keppie, J., Smith, H. & Harris-Smith, P. W. (1953). *Brit. J. exp. Path.* (in the Press).

The Effect of Plasma β-Lipoprotein Concentration on the Action of Injected Heparin in the Rabbit. By A. Comfort.* (*Department of Zoology, University College, London, W.C.* 1)

Intravenous injection of heparin in rabbits made lipaemic by lanolin feeding produces a large increase in the anodic mobility of the β-lipoprotein front at pH 8·6. The same effect is produced *in vitro* by addition of plasma from heparin-injected animals, but not by heparin itself. It is therefore presumably due to heparin 'clearing factor' (Comfort, 1953).

We have followed the response of rabbits to injected heparin (*a*) during the decline of lipaemia after cessation of several months' lanolin feeding, (*b*) during an acute episode of lipaemia produced by one week of lanolin feeding. Blood was drawn before and 10 min. after injection of 1000 units of heparin (Pularin Evans) into the ear vein in young does (2–3 kg.), and examined by paper electrophoresis and staining with Sudan black B, as previously described by us. Serum cholesterol was estimated in duplicate on the pre-injection sample by the method of Abell, Levy, Brodie & Kendall (1952).

At serum cholesterol levels > 1 mg./ml. heparin produced an increase in β-lipoprotein migration distance in all cases. The percentage increase varied greatly, both between animals, and in the same animal on different occasions. The magnitude of the increase bore no simple relation to blood cholesterol level or to dose/body weight.

At serum cholesterol levels < 1 mg./ml. approx., injected heparin either failed to affect β-lipoprotein mobility at all, or reduced it compared with parallel

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controls. A very small increase in mobility could usually be produced at these levels by adding guinea-pig 'clearing factor', but not by increasing the dose of heparin. The slight action of heparin on the α_2-lipoprotein did not appear to be affected. The effect is not due to failure of 'clearing factor' production in the rabbit at low serum lipoid levels, since the heparinized plasma samples were active when tested against lipaemic rabbit plasma. The appearance of the electrophoretic strips suggests rather that this is a concentration effect than that the changing behaviour of rabbit lipoproteins with increasing lipaemia is due to the appearance of a heparin-sensitive component.

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Antithyroid Activity of some *S*-Substituted 2-Mercaptoglyoxalines. By A. Lawson and C. E. Searle.* (*Royal Free Hospital School of Medicine, 8 Hunter Street, London, W.C. 1*)

Antithyroid drugs widely used in recent years for the treatment of hyperthyroidism have been 2-thiouracil, its 6-methyl and 6-propyl derivatives, and 2-mercapto-1-methylglyoxaline.

These substances are usually assumed to owe their activity to their rapid reduction of iodine to iodide, this process interfering with the iodination of tyrosine in the thyroid protein and hence with the synthesis of the thyroid hormone. A free —SH group has therefore been regarded as essential. 2-Carbethoxythio-1-methylglyoxaline was however shown to have antithyroid activity in the rat comparable to that of the parent thiol (Lawson, Rimington & Searle, 1951) and has since been used clinically (Doniach, 1953; Poate, 1953).

This substance probably owes its activity to an *in vivo* hydrolysis to the active thiol, and some similar compounds, 2-carbomethoxythio-, 2-carbobenzyloxythio- and 2-hippurylthio-1-methylglyoxaline, and di(1-methylglyoxaline) 2:2-dithiolcarbonate, all show high activity in the rat.

It had been expected that 2-alkylthioglyoxalines would be quite devoid of activity, but some slight activity had been observed in 2-benzylthioglyoxaline (Searle, Lawson & Hemmings, 1950). Some *S*-benzyl- and *S*-methyl-derivatives of active 2-mercaptoglyoxalines were therefore examined by the same method but at rather larger dosages (25–50 mg./kg. body weight), and in several cases over 50% depressions of radio-iodine uptake over 4 hr. were observed. 2-Benzylthio-4-methylglyoxaline in this test appears to be about one tenth as active as 2-mercapto-4-methylglyoxaline itself.

Whilst the possibility exists that some thiol may be produced in the body by removal of the methyl or benzyl groups, the usual result of *in vitro* reductions is fission of the glyoxaline-sulphur bond. Since from the action of iodine on 2-benzylthio-4-methylglyoxaline a 5-iodo-derivative has been obtained, *in vivo* iodination may account for the activity of these compounds.

* I.C.I. Research Fellow.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **323rd Meeting** *of the Biochemical Society was held in the Department of Biochemistry, St Mary's Hospital Medical School, Paddington, W. 2, on Saturday, November 14th, 1953, at 11.30 a.m., when the following papers were read:*

COMMUNICATIONS

The Metabolism of *iso*Propylbenzene (Cumene). By D. Robinson, J. N. Smith and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Cumene when fed to rabbits causes the excretion of considerable amounts of conjugated glucuronic acid (68% at 450 mg./kg.) (Smith, Smithies & Williams, 1953). Paper chromatography of the glucuronide fraction revealed the presence of three glucuronides which have been identified and isolated as triacetyl methyl esters. These were the glucuronides of α-phenylpropionic acid (hydratropic acid), 2-phenylpropan-1-ol (hydratropyl alcohol) and 2-phenylpropan-2-ol. Cumene is thus oxidized at each carbon atom of the side chain thus:

$$PhCH(CH_3)_2 \rightarrow PhC(OH).(CH_3)_2 + PhCH(CH_3).CH_2OH + PhCH(CH_3).COOH.$$

Both hydratropic acid and hydratropyl alcohol contain an asymmetric carbon atom. Preliminary experiments show that both forms of hydratropic acid are formed biologically from cumene, with the (+) form in excess.

Gentle heating of cumene urine with dilute acid yields α-methylstyrene ($PhC(CH_3):CH_2$) which accounts for at least 26% of the cumene fed. This appears to arise from 2-phenylpropyl-2-glucuronide.

Calculations based on the yields of hydratropic acid, hydratropyl glucuronide and α-methylstyrene, suggest that at least 15% of the dose of cumene is oxidized to hydratropic acid, 15% to hydratropyl alcohol and 26% to 2-phenylpropan-2-ol. Thus it appears that the α-carbon of cumene is more readily oxidized *in vivo* than the β- (or ω-) carbon atoms.

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Presence of Cytochrome in an Obligate Anaerobe. By J. R. Postgate (introduced by V. A. Knivett). (*Chemical Research Laboratory, Teddington*)

The sulphate-reducing bacteria (*Desulphovibrio desulphuricans*) are anaerobes so strict that small populations do not grow in the absence of air unless a reducing agent is added (Grossman & Postgate, 1953). Nevertheless, spectroscopic examination of two halophilic and two non-halophilic strains (>14 mg. dry wt./ml.) showed large amounts of reversibly oxidizable cytochrome-like pigments. More detailed examination of one strain ('Hildenborough') showed the following points:

(1) Visible bands at 525 and 553 mμ. disappeared on shaking in air and returned on standing or passing in H_2. A band at 630 mμ. was unaffected both by this treatment and by more drastic oxidation or reduction.

(2) The 'reversible oxidation' of the 525 and 553 mμ. bands was not affected by Na_2S, KCN or CO.

(3) Spectrophotometric examination of cells by Barer's (1953) method showed the visible peaks mentioned and a Soret peak at 418 mμ.

(4) Preparation of cell-free extracts indicates the presence of two pigments: one giving the 630 mμ. absorption and the other responsible for the absorptions at 418, 525 and 553 mμ. The latter has a peak at 410 mμ. only when oxidized and in several ways resembles the cytochrome *c* of yeast and muscle.

(5) The *c*-component leaks out of cells treated with lethal amounts of cetyltrimethylammonium bromide, though the 630 mμ. component does not. Such cells are unable to reduce the *c*-component with organic hydrogen donors but can still do so with gaseous hydrogen.

(6) Cell-free hydrogenase preparations do not reduce the cell-free *c*-component; neither intact nor damaged cell preparations reduce muscle cytochrome *c*.

Thus these bacteria contain a pigment resembling, though not identical with, cytochrome *c*, but the lack of inhibition by KCN, etc., shows that normal cytochrome oxidase is absent. The metabolic function of this pigment is obscure; it may well be

concerned in the metabolism of gaseous H_2, but attempts to demonstrate a role in sulphate reduction have been unsuccessful. Its presence, however, demonstrates that the absence of cytochromes from obligate anaerobes can no longer be taken as axiomatic.

I am indebted to Dr Barer for the use of his methods; to Dr Hartree for certain confirmatory examinations and invaluable advice; to Dr Lascelles who first saw these pigments and to numerous other workers for advice and encouragement.

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Metabolism of Mammalian Tissues with Little or no Added Aqueous Phase. By H. McIlwain and R. Rodnight. (*Biochemical Laboratories, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

In tissue-slice experiments the conventional quantity of aqueous medium (for example, 2·5 ml./50 mg. moist tissue) limits certain measurements and is an expedient often acceptable but sometimes adopted unnecessarily. Seeking to minimize added fluid, cerebral cortex, kidney cortex, liver and spleen of rat, guinea pig and rabbit were cut, weighed and placed on frames which supported them in conical manometric vessels with large necks and eccentric 'alkali wells'. Respiration was measured in moist oxygen by ordinary manometers and products determined in tissue and washings at the end of experiments. Substrates were in some cases provided by brief contact or incubation with physiological salines containing them.

Respiratory rates found with cerebral tissues were between 68 and 120, with kidney cortex 127 to 148, and with liver about 60 μmoles O_2/g. fresh weight/hr. Respiration did not persist for more than about 30 min. at the higher rates unless substrate was provided (e.g. as 0·05 ml. 0·1 M solution). Rates with glucose and minimal fluid were frequently higher than in glucose-phosphate salines under normal conditions. Further addition of such salines to immerse cerebral or kidney cortex previously respiring with minimal fluid lowered respiratory rates by 20–45 %. Rates with cerebral tissues and minimal fluid containing glutamate, succinate or lactate were also high.

In experiments with guinea-pig cerebral cortex and added glucose, moist tissue converted into lactic acid about 15 % of the glucose utilized. Tissue in 50 vol. saline converted 62 % into lactic acid in the same time. The former value is much closer to the behaviour of the brain *in situ*. Probably the large fluid volumes ordinarily used *in vitro* allow accumulation of material which would otherwise be further metabolized. This can be valuable in identifying such materials but limits quantitative comparison with the tissue *in vivo*. Lactate oxidation by separated cerebral tissues increases greatly between 2 and 20 mM.

Respiration of slices of cerebral cortex moist after exposure to glucose saline could be increased by suitable electrical pulses. pH does not fall seriously during such experiments.

Metabolism of Mammalian Tissue-fragments Suspended in Non-aqueous Fluids. By R. Rodnight and H. McIlwain. (*Biochemical Laboratories, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

As a part of the study of tissue-metabolism with minimal aqueous media, measurements have been made with moist sliced tissues mechanically supported and immersed in non-aqueous fluids. It was intended in this way to supply to the tissue substrates and inhibitory or stimulating agents, and to remove metabolic products. The non-aqueous fluids were chosen on the basis of physical properties and previous physiological practice. They included olive oil, light liquid paraffin, and silicone fluids.

Tissues were cut, weighed, mounted and transferred to conical manometric vessels with sidearms, wide mouths and a displaced 'alkali well'. A modified manometer permitted the non-aqueous fluid already equilibrated with the moist gas phase to be pipetted to the vessel without exposure to air. (This was necessary because the high solubility of atmospheric gases in the oils otherwise led to long equilibration periods.)

Oxygen uptake of slices of brain, kidney, liver and spleen under these conditions were again as high as or higher than those obtained using conventional volumes of a saline medium. Initial rates were adequately maintained when substrate was supplied.

This was done with small volumes of adhering saline or by a fine suspension of solid glucose in the oil.

Cerebral cortex slices, moist with glucose salines and respiring in liquid paraffin, responded to alternating condenser pulses by a 50–75% increase in respiratory rate. This increase could be reduced by the incorporation of 0·1 mM phenobarbitone or butobarbitone or 1 mM atropine in the oil. Addition of 25 μM dinitrophenol dissolved in liquid paraffin to slices respiring in that medium resulted in an increase in respiratory rate similar to that found when the drug is added to cerebral tissue respiring in saline.

An Examination of Human Serum for the Presence of Arylsulphatase. By K. S. DODGSON and B. SPENCER. (*Physiology Institute, University of Wales, Cardiff*)

Notable variations in human serum β-glucuronidase activity occur during pregnancy, oestrogen and ACTH therapy and in certain malignant states (see Fishman, 1951). A study of serum arylsulphatase has now been made in order to ascertain whether a similar pattern of behaviour is shown by this enzyme.

Conflicting reports have appeared concerning the presence of arylsulphatase in serum. Abbott (1947) could not detect activity with potassium phenylsulphate as substrate, but Huggins & Smith (1947), using potassium *p*-nitrophenylsulphate, observed activity of 30–1500 units/100 ml. (1 unit being equivalent to 1 μg. of the phenol liberated/hr.). Both authors used conditions of assay which were optimum for the fungal enzyme, and since there are considerable differences between arylsulphatases from different sources (Dodgson, Lewis & Spencer, 1953), it was first necessary to study the kinetics of the human enzyme.

Suspensions of human liver had optimum activity at pH 6·9 in M acetate with a concentration of potassium *p*-acetylphenylsulphate, the independent substrate chosen (Dodgson & Spencer, 1953; Dodgson, Spencer & Thomas, 1953), of 0·005M. The figures for citrate and phosphate buffers were pH 7·1 and 7·3 and 0·003 and 0·008M respectively, but the activity in acetate was approximately four times greater than in the other buffers. Heparin and oxalate did not affect the enzyme when present in the normal amounts required to prevent blood coagulation but citrate and fluoride caused 3–5% inhibition. Human urine inhibited the enzyme by approximately 35% when present in 40% concentration but a similar amount of serum was without effect.

Using potassium *p*-acetylphenylsulphate in acetate under the optimum conditions and potassium *p*-nitrophenylsulphate according to the instructions of Huggins & Smith, a large number of normal and pathological sera and plasmas and sera from women in the later stages of pregnancy were assayed. With a 20–24 hr. incubation period activity was less than 20 units/100 ml. by either method and sera of ox, sheep, dog, rat and rabbit and human urine assayed under the same conditions showed no greater activity. The highest values recorded are only twice the sensitivities of the respective methods.

On the addition of alkali to acetate, potassium *p*-nitrophenylsulphate and serum, as in the assay method, liberation of *p*-nitrophenol occurs; the rate of reaction depending on temperature and strength of alkali. The factor responsible was present in all mammalian sera tested and was thermostable and non-dialysable. It did not attack the sulphates of phenol, *p*-hydroxyacetophenone and phenolphthalein and was absent from perfused rat tissues and urine. The mechanism of action appears to be a catalysis of the alkaline hydrolysis of *p*-nitrophenylsulphate.

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Variation of the Antigenic Structure of Certain Bacteria with Temperature of Incubation and Ionic Composition of the Medium. By B. W. Lacey (introduced by N. F. Maclagan). (*Department of Bacteriology, Westminster Medical School, S.W.* 1)

Freshly isolated strains of the *Haemophilus pertussis—parapertussis—bronchisepticus* group may grow in either of two contrasting antigenic states (or modes). Which mode develops depends on the conditions of growth, especially temperature of incubation, ionic composition of the medium and presence of surface-active substances. The influence of the following ions has been examined: (1) Li^+, Na^+, K^+, NH_4^+; (2) Mg^{2+}, Ca^{2+}, Sr^{2+}; (3) Cl^-, Br^-, I^-, NO_3^-, formate; (4) acetate, lactate, fumarate, malonate, succinate, aconitate, thiocyanate; (5) sulphate, selenate, butyrate and higher homologues, glutarate and higher homologues, trichloroacetate, etc. With *H. pertussis* at 35°, salts of type (1)+(3) tend to induce one mode (*X*); salts of type (1)+(4) are near neutral; all other types tend to induce the other mode (*C*). The influence of a salt in a given system appears to be proportional to its specific modulating power, concentration and conductance ratio. Hence the overall influence of a mixture of salts depends on the nature and relative effective concentrations of its components and not on its total ionic strength. The change of mode with temperature and change in the proportion of Na^+, Mg^{2+}, Cl^- and SO_4^{2-} has been examined in members of the three species. Three-dimensional diagrams of the results with all three species have a similar shape. But at the same temperature a different ionic mixture is needed to induce an equivalent antigenic state in the different species, while, with a given ionic mixture, a different temperature is needed. The results have also been arranged as a nomogram in which the distance of any salt from a selected antigenic state is proportional to the square root of its modulating power. From this the mode of growth can be calculated given the species, temperature, salt concentrations and conductance ratios.

The Dissociation Constants of the Magnesium Salts of Phosphoric Acid, Glucose 1-phosphoric Acid and Glycerol 2-phosphoric Acid. By H. B. Clarke, D. C. Cusworth and S. P. Datta. (*Department of Biochemistry, University College, London*)

As many enzymes concerned with phosphate metabolism are known to be magnesium activated, and as a number of magnesium salts of divalent acids such as the sulphate, malonate and orthophosphate, are known not to be fully dissociated in aqueous solution, it is of interest to examine the extent of dissociation of the magnesium salts of some phosphate esters and of phosphoric acid itself.

Phosphate or ester phosphate buffers containing $MgCl_2$ were examined potentiometrically in cells without liquid junction of the type

$$\underset{}{PtH_2} \,|\, \underset{m_a}{MHA},\ \underset{m_b}{M_2A},\ \underset{m_s}{MgCl_2} \,|\, AgCl.Ag$$

The e.m.f. of such a cell is given by

$$E = E_0 - \frac{2{\cdot}3026RT}{F} \log a_{H^+} . a_{Cl^-} .$$

From this equation in terms of molalities and the approximation for the activity coefficient proposed by Guntelberg (1926), we have

$$\log m_{H^+} = \frac{-(E-E_0)F}{2{\cdot}3026RT} - \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}},$$

which substituted in the expression for the pK of the acid, HA^-:

$$-\log k_a = -\log \frac{m_{H^+} . m_{A^{2-}}}{m_{HA^-}} + \frac{4AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}},$$

gives

$$\log m_{A^{2-}} = \log k_a + \log m_{HA^-} + \frac{(E-E_0)F}{2{\cdot}3026RT} + \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}},$$

where

$$m_{HA^-} = m_a - m_{H^+} . \qquad (1)$$

The ionic strength of the above system is given by

$$I = m_a - m_b + 3m_s - 2m_{H^+} + 4m_{A^{2-}} . \qquad (2)$$

It is apparent that equations (1) and (2) must be iterated to obtain final values of $m_{A^{2-}}$ and I, which can then be used to obtain $K_s = \frac{a_{Mg^{2+}} . a_{A^{2-}}}{a_{MgA}}$ by the equation

$$-\log K_s + \beta I = -\log m_{Mg^{2+}} - \log m_{A^{2-}} + \log m_{MgA} + \frac{8AI^{\frac{1}{2}}}{1+I^{\frac{1}{2}}} \qquad (3)$$

where $m_{MgA} = m_b + m_{H^+} - m_{A^{2-}}$

and $m_{Mg^{2+}} = m_s - m_{MgA} .$

The right-hand side of equation (3) is plotted against I to give a straight line, and the intercept at $I=0$ obtained by the method of least squares.

Measurements were made at 10° intervals from 10 to 50°. The dissociation constants varied

with the absolute temperature according to the equation

$$-\log K = \frac{A}{T} - D + CT,$$

from which the thermodynamic quantities associated with the dissociations were calculated.

At 37° the thermodynamic pK's are $2{\cdot}801 \pm 0{\cdot}003$, $2{\cdot}575 \pm 0{\cdot}007$ and $2{\cdot}595 \pm 0{\cdot}015$ for magnesium orthophosphate, magnesium glucose 1-phosphate, magnesium glycerol 2-phosphate respectively. The values of the constants A, D and C are 1305, 8·2 and 0·022 for magnesium orthophosphate, 1288, 8·3 and 0·022 for magnesium glucose 1-phosphate, and 1058, 7·1 and 0·020 for magnesium glycerol 2-phosphate.

The values of k_a used above were those of Bates & Acree (1943) in the case of phosphoric acid, and Ashby, Crook & Datta (1953) in the case of glucose 1-phosphoric acid, and glycerol 2-phosphoric acid.

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Stabilizing Linkages in Tendon. By D. S. Jackson (*Rheumatism Research Centre, Manchester University*)

It has been suggested (Jackson, 1953) that chondroitin sulphuric acid (C.S.A.) plays an important part in stabilizing the structure of tendon. This communication is concerned with a study of the types of linkages which may be involved in this stabilization, using the shrinkage temperature as a measure of stability.

Gustavson (1949) has pointed out that an evaluation of the relative importance of electrovalent and hydrogen bonds can be obtained by measuring the shrinkage temperature of collagen after treatment specific for these two linkages.

β-Naphthalenesulphonic acid eliminates salt-like cross-links (Gustavson, 1942) and concentrated calcium chloride solutions affect hydrogen bonds (Theis, 1946). In addition to these two reagents, the effect of periodic acid has also been studied.

These methods have been applied to native tendon, hyaluronidase-treated tendon, and collagen reconstituted from acetic acid solution with salt and C.S.A., and their shrinkage temperatures determined, before and after treatment, in increasing concentrations of calcium chloride (0–2·7 M).

It was found that before treatment native tendon and C.S.A.-precipitated collagen behaved in a similar way, as did hyaluronidase-treated tendon and salt-precipitated collagen, indicating that the stability of tendon and precipitated collagen is conditioned by the presence of C.S.A.

Periodic acid treatment caused a considerably greater fall in the shrinkage temperature of native tendon in water than did hyaluronidase (24° as compared with 10–12°), which may indicate the presence of a further stabilizing mucopoly-saccharide or mucoprotein.

Treatment with β-naphthalenesulphonic acid following either hyaluronidase or periodic acid, caused a further fall in shrinkage temperature of 4° compared with the 10° fall caused by treatment with β-naphthalenesulphonic acid alone. The result is interpreted as meaning that 60 % of the salt-like cross links are between C.S.A. and collagen.

On the basis of these results the stabilizing bonds present in tendon are (as % of total) for mucoprotein and/or C.S.A.—collagen, salt-like bonds 13, hydrogen bonds 38 and for collagen—collagen, salt-like bonds 9, hydrogen bonds 40.

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The Occurrence of Lactose in the Spleen Cerebrosides of a Case of Gaucher's Disease. By D. V. Parke. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Halliday, Deuel, Tragerman & Ward (1940) have shown that the accumulated cerebrosides of Gaucher's disease were glucosidocerebrosides, but Lieb & Günther (1941) showed that both glucosidocerebrosides and the normally occurring galactocerebrosides may be found in this condition. The lipids of the spleen removed from a 31-year-old woman with Gaucher's disease were fractionated by extraction with acetone, petroleum ether, and methanol-$CHCl_3$. In this way the tissue was shown to contain 72·8 % (w/w) of moisture, 1·1 % of neutral fat and cholesterol, 2·1 % of phospholipids, and 3·4 % of cerebrosides. The cerebroside fraction was purified by dissolving in pyridine and precipi-

tating with acetone followed by recrystallization from methanol-$CHCl_3$ (Halliday *et al.* 1940). This material was then hydrolysed in 2N-H_2SO_4 at 100° for 3 hr., cooled, and adjusted to pH 4 by addition of a saturated solution of $Ba(OH)_2$. The filtered solution was concentrated *in vacuo* over P_2O_5 at 20°. Chromatography of this material in ethyl acetate-pyridine-water (2:1:2), and *n*-butanol-water at 37° showed the presence of glucose and galactose in approximately equal amounts, and another, weaker spot, with the same R_F values as lactose. This spot disappeared on incubation of the hydrolysed cerebroside with a lactase preparation of *E. coli* (Monod, Torriani & Gribetz, 1948). No lactose could be demonstrated in mixtures of glucose and galactose subjected to the treatment given to hydrolyse the cerebroside.

On keeping a solution of the hydrolysed cerebroside in aqueous ethanol at 0° for several weeks, crystals separated. These (3 mg.) were recrystallized from aqueous ethanol, and had m.p. 200–205° (decomp.) not depressing that of authentic lactose (202° decomp.). This material was chromatographically identical with lactose and readily distinguishable from melibiose, and on hydrolysis gave only glucose plus galactose.

Lactose could not be demonstrated in the hydrolysed cerebroside isolated from the same spleen kept for several weeks in formalin, although both glucose and galactose were present. This preserved material showed a considerable increase over the fresh tissue in the acetone soluble lipids, with a concomitant decrease in the cerebroside fraction.

The increased phospholipid content of the fresh spleen was due solely to an increase of sphingomyelin (1·6 %, w/w), which is usually associated with Niemann-Pick's disease, but not with Gaucher's disease.

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The Sugar-containing Lipids of Gaucher's Disease. By L. I. Woolf. (*The Hospital for Sick Children, Great Ormond Street, London, W.C.* 1)

In Gaucher's disease the spleen, etc., become swollen with deposits of a lipid long identified as a cerebroside. Aghion (1934) showed that glucose, not galactose, was the sugar component of the lipid. Klenk (1940, 1942) identified the sugar as pure glucose by its fermentability, osazone, etc., though it gave a positive Seliwanoff reaction indicating the presence of a ketose; however, Lieb (1942) and Uzman (1951) found galactose as the only sugar present in the lipid. The application of paper chromatography to the problem is reported here.

The material comprised the spleens of (*a*) an adult, (*b*) a child, (*c*) an infant, all three having Gaucher's disease, and (*d*) the brain of a child with an unidentified lipidosis. These were obtained through the kindness of Dr E. Neumark (*a*), Dr M. Bodian (*b* and *c*), and Dr L. Crome (*d*). The lipid was extracted by the technique of Folch, Ascoli, Lees, Meath & Le Baron (1951).

(1) *Complete hydrolysis.* The lipid was emulsified with sodium lauryl sulphate (Brand & Sperry, 1941) and heated with 3N-H_2SO_4 for 10 min. at 100°. Lipids, anions and cations were removed (Woolf, 1953) and the solution examined by paper chromatography, using aniline phthalate as reagent for aldoses and naphthoresorcinol with trichloroacetic acid for ketoses:

(*a*) gave aldose-reacting spots with the R_F values of glucose (0·25 % of fresh spleen), galactose (0·04 %) and a ketose-reacting spot with the R_F of fructose (0·04 %). After fermentation with yeast only the galactose spot remained; (*b*) gave spots corresponding to glucose (0·24 %) and galactose (0·04 %); (*c*) gave spots corresponding to glucose and a trace of fructose; (*d*) gave a galactose spot and a faint spot with the R_F value and colour reaction of fructose. At this point lactose, galactose and glucose were independently reported in spleen (*a*) (Parke, 1953).

(2) *Partial hydrolysis* was carried out omitting sodium lauryl sulphate and using N-H_2SO_4 at 100° for 4 min. The yield of sugar was much reduced, particularly fructose and galactose in case (*a*).

(*a*) Two sugars with the R_F values and colour reactions of lactose and sucrose appeared. Fermentation with yeast eliminated sucrose, glucose and fructose spots. No arabinose was detected (cf. Hutt, Malkin, Poole & Watt, 1950).

(*b*) Lactose, galactose and glucose appeared.

(*c*) Sucrose, fructose and galactose appeared (identification by R_F values, colour reactions and fermentability).

In both brain and spleen the lipid containing sucrose (and therefore fructose) is more labile and more soluble in ether than the lipids containing glucose, galactose or lactose.

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Folic Acid as Coenzyme for the Interconversion of Serine and Glycine. By R. L. Blakley.*
(*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

The biosynthesis of serine from glycine and formate has been shown to occur *in vivo* (Sakami, 1948) and in cell-free preparations of pigeon and chicken liver (Deodhar & Sakami, 1953). The reverse enzymic breakdown of serine to glycine and formaldehyde has been described by Vilenkina (1952), who reported reactivation of the dialysed enzyme by boiled liver or yeast extract or folic acid.

Conversion of serine into glycine has been studied by incubating [2-^{14}C]-L-serine (0·02 M) anaerobically in presence of a soluble enzyme from pigeon liver. Carrier glycine was added at the end of the experiment, and subsequently separated chromatographically as its dinitrophenyl derivative and counted.

Using well-dialysed enzyme preparations, a considerable activating effect of folic acid was obtained. Under optimum conditions 10–20% of the serine was converted into glycine in 6 hr., and an approximately equivalent amount of formaldehyde appeared in the solution.

Since there is evidence that the above reaction is reversible, the enzyme should catalyse the interconversion of serine and glycine:

$$CH_2OH.CHNH_2.COOH \rightleftharpoons CH_2NH_2.COOH + HCHO.$$

Evidence for a freely reversible reaction of this type was obtained by incubating [2-^{14}C]-glycine (0·02 M) and L-serine (0·05 M) in presence of the enzyme preparation. Radioactive isotope was readily incorporated into the serine and degradation of the isolated serine demonstrated that the isotope was almost exclusively located in the α-carbon.

Soluble enzyme preparations catalysing this reversible reaction were also activated by addition of folic acid and boiled yeast or liver extract, maximum activity being obtained in presence of both folic acid and yeast extract. The requirement of the enzyme for folic acid and yeast extract varies with the treatment of the enzyme, being greatest after the enzyme preparation has been dialysed for 1–2 days. With such preparations 70-fold increases of activity have been obtained on addition of folic acid ($1·5 \times 10^{-4}$ M).

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* Australian National University scholar.

DEMONSTRATIONS

Electrophoresis in Agar with the Bridge Unit. By E. KAWERAU. (*Department of Chemical Pathology, St Mary's Hospital, London, W.* 2)

Gaucher's Disease; A Rare Disorder of Lipoid Storage. By E. NEUMARK (introduced by R. T. WILLIAMS). (*From the Department of Morbid Anatomy, St Mary's Hospital Medical School, London, W.* 2)

The material was obtained from a Jewish woman, aged 31 years, who had abdominal pain, enlargement of liver and spleen, pingueculae (bright yellow areas in the conjunctivae) and a generalized slight yellow discoloration of the skin. Sternal marrow biopsy (Leitner, Britton & Neumark, 1949) showed large atypical cells with vacuolated cytoplasm. Blood counts showed moderate anaemia and marked thrombocytopenia. Radiographs of both femora showed the characteristic hock-bottle shape of the lower half. As a palliative measure (Thannhauser, 1950) splenectomy was carried out. The spleen weighed 2600 g. and was studded with large and small nodules. Smear preparations from the cut surface showed the typical Gaucher cells, which are 30–60 μ. in diameter, have small eccentric nuclei and foamy pale cytoplasm looking like crushed tissue paper. Sections showed extensive replacement of the splenic architecture by groups and alveoli of Gaucher cells. The nodules corresponded to necrotic areas often surrounded by haemorrhages. Sudan III did not stain the cytoplasm. Periodic acid and Schiff's reagent stained it a light mauve, which is suggestive of a muco-polysaccharide. Preparations from lymph nodes found near the hilum of the spleen showed similar appearances. Chemical analysis of the spleen was carried out by D. V. Parke of the Department of Biochemistry and by L. I. Woolf of the Hospital for Sick Children, Great Ormond Street, London.

Clinical and morphological features in this case were similar to those of a case described by Kettle (1920) and preserved in the museum. In all cases of Gaucher's disease the lesions are practically constant, so that although the material available is comparatively small, the disease is quite clearly established.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **324th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, The London Hospital Medical College, Turner Street, London, E. 1, on Saturday, 12 December* 1953, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Diffusible Peptide-like and Glycosidic Constituents of Italian Rye Grass. By R. L. M. Synge and J. C. Wood. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Data collated by Pardee (1951) suggest that phenol should be a useful selective solvent for extracting peptides. In attempts to purify the diffusible neutral 'bound amino acid' of Italian rye grass (fraction 3-*M*, Synge, 1951; N, approx. 0·1% of dry matter of fraction) a 5-unit counter-current distribution with phenol-water yielded most of the nitrogenous material in the phenol-rich extracts while eliminating higher saccharides and most of the monosaccharide present. Doing counter-current distribution before electrophoretic fractionation increased the amount of material conveniently handled.

Besides peptide-like material, this procedure concentrated water-soluble glycosidic material. Acid hydrolysis (N-HCl, 2 hr. at 100°) liberated water-insoluble material (partly soluble in ether, entirely in ethanol) and glucose (with smaller amounts of galactose and rhamnose). A preparation from spring grass gave: reducing sugar liberated (as glucose), 0·41%; ether-extractable and water-insoluble material, 0·64% of dry matter of original grass. Corresponding figures for a late flowering stage were 0·10 and 0·20%, fluorescent and pigmented materials being likewise present in smaller amounts. These glycosides may be lignin precursors; Manskaya & Bardinskaya (1952) noted similar changes during lignification.

Preparations were further fractionated by partition chromatography (phenol-water; phenol phase stationary) on dichlorodimethylsilane-treated kieselguhr (Howard & Martin, 1950). The peptide-like material (detected according to Rydon & Smith (1952), by N determination, etc.) emerged from the chromatogram without well-defined peaks. However, rechromatography of any cut gave a peak in the corresponding position, demonstrating effective fractionation of heterogeneous material. Acid hydrolysis (as above) broke few peptide bonds and permitted removal of the aglycone fraction. On chromatography reducing sugar was then concentrated in early effluent fractions, and peptide material, whose chromatographic behaviour was not much affected by the acid treatment, was obtained less contaminated in later fractions (N, 1–6% of dry matter). Cuts from phenol-water chromatograms have been further fractionated on kieselguhr chromatograms (*n*-butanol-acetic acid-water systems); some cuts also give well-defined fractions on paper chromatography with water or aqueous KCl. A complicated series of compounds is thus revealed.

Phenol likewise selectively extracts the acidic peptide-like material of fraction 4-A (Synge, 1951). On further fractionation by paper electrophoresis, the Rydon test revealed a complicated picture (cf. Turba & Esser, 1953). We are further exploring the use of phenol for extracting peptide material from indiffusible grass fractions.

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Porphyrins in the Urine of Normal Men. By A. Comfort and M. Weatherall. (*Department of Pharmacology, London Hospital Medical College, London, E.* 1)

The porphyrins present in the urine of healthy young adult males have been extracted by adsorption at pH 2–2·5 on talc, esterified with methanolic sulphuric acid and chromatographed from benzene with increasing concentrations of chloroform and finally with chloroform containing methanol on alumina columns. The fractions were examined by paper chromatography with lutidine-water after acid hydrolysis (Nicholas & Rimington, 1951). Seven batches, of 2·5–29 l., were examined. All contained predominantly coproporphyrin. All but one yielded at least two other porphyrin bands on

the alumina column. These bands were less easily eluted than coproporphyrin and on paper chromatography gave mixtures of porphyrins with up to five constituents all with R_F values lower than that of coproporphyrin (mean 0·52, 0·43, 0·33, 0·13 and 0·07; coproporphyrin = 0·67, uroporphyrin = 0·07 on these papers). The properties of these fractions appear similar to some of those recently reported in much larger amounts in porphyric urines.

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Intramanometric Dialysis. By H. LASER. (*Molteno Institute, University of Cambridge*)

The oxidation of ethanol by catalase has so far only been obtained in presence of an enzymic system, like amino acid oxidase, xanthine oxidase or notatin + their specific substrates, the oxidation of which provides a continuous supply of hydrogen peroxide (Keilin & Hartree, 1936, 1945). When H_2O_2 as such is added to catalase + ethanol, only an insignificant fraction of it is used in peroxidatic oxidation, the main portion being catalytically decomposed to H_2O and O_2.

In order to obtain a greater efficiency in peroxidatic oxidation of ethanol by catalase on adding H_2O_2 as such, the following conditions had to be fulfilled: H_2O_2 had to be introduced (1) continuously for a certain period of time, (2) at a predetermined measurable rate, (3) in variable concentrations. This, it was thought, could be obtained by admitting the peroxide by dialysis.

A manometer vessel has therefore been devised which fulfils these conditions. It consists of an upper and a lower part which are divided by a cellophan membrane. The upper part contains the enzyme while the lower part is filled with a buffer solution. This part is provided with a Dixon-Keilin tap of the type introduced by these authors in their method for the determination of the respiratory quotient (Dixon & Keilin, 1933). Its cavity contains the substance to be dialysed, e.g. H_2O_2. On turning the tap 'on' so as to connect it with the lower part of the vessel H_2O_2 is released into the buffer solution and dialysis into the upper part of the vessel commences. In this way the rate of diffusion of H_2O_2 has been measured manometrically by the rate of liberation of O_2 by catalase. The rate of diffusion, as was to be expected, decreases exponentially and is proportional to the H_2O_2 concentration. The rate of decomposition of H_2O_2 was within wide limits independent of the catalase concentration. The application of intramanometric dialysis for the study of other biological problems will be briefly discussed.

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Peroxidatic Activity of Catalase. By H. LASER. (*Molteno Institute, University of Cambridge*)

The peroxidatic activity of catalase has been studied by means of intramanometric dialysis (see previous communication), whereby H_2O_2 of known concentration is introduced at a predetermined rate into a buffer solution containing ethanol and catalase. If peroxidatic oxidation of ethanol to aldehyde takes place, less O_2 is liberated from peroxide than in the control in absence of ethanol. The greater the difference the more the aldehyde that has been formed. Thus the oxygen liberated in presence of ethanol under given experimental conditions expressed as percentage of that of the control is a direct measure of the efficiency of peroxidatic oxidation.

By varying the concentrations of catalase (2×10^{-5}–2×10^{-10} M), of ethanol (0·2–8·0 %) and of initial H_2O_2 in the lower chamber ($0{\cdot}25$–$2{\cdot}0 \times 10^{-3}$ M) the following results have been obtained: The efficiency of peroxidatic oxidation *increases* (1) with rising catalase concentration at constant ethanol concentration, reaching 70–80 % with a 2×10^{-5} M catalase; (2) with rising ethanol concentration at constant catalase concentration. It *decreases* with rising H_2O_2 concentration at constant catalase and ethanol concentrations. It is concluded, in agreement with the view of Chance (1949, 1950), that both H_2O_2 and ethanol compete for the H_2O_2-catalase complex I. Peroxidatic as opposed to catalatic activity of catalase is favoured by a low concentration of free H_2O_2 relative to that of ethanol.

The formation of aldehyde, besides its characteristic smell, has been confirmed by a brown decoloration of KOH-soaked filter paper (polymerization) and by Schiff's reagent.

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A Histochemical Study of the Specificity of Phosphatases. By G. H. Bourne (introduced by F. L. Warren). (*Department of Histology, London Hospital Medical College, London, E.* 1)

If the ability of alcohol or acetone fixed tissues to hydrolyse various phosphate esters is studied histochemically it can be seen that the distribution of dephosphorylating activity differs with the ester used. The distribution of dephosphorylating activity of sugar phosphates such as those of fructose, sorbose and galactose and also of vitamin K phosphate is similar to that obtained with glycerophosphate and this suggests that these substances are hydrolysed by the non-specific phosphatases.

However, the distribution of dephosphorylating activity for other phosphate esters differs considerably from that obtained with those mentioned above. The other esters studied were riboflavin 5-phosphate, pyridoxal phosphate, aneurin pyrophosphate, tetrasodium 3:4′-dihydroxy-4-methoxychalkone diphosphate, tetrasodium 2′:3-dihydroxy-4:4′-dimethoxychalkone phosphate, oestrone 3-phosphate, tetrasodium stilboestrol diphosphate, tetrasodium hexoestrol diphosphate.

With the latter group of esters the dephosphorylating activity is much greater in the nuclei, and particularly in the nucleoli, than with the first group. In the testis the sperm heads were very positive with the chalkone phosphates.

The hydrolysis of all the esters was tested at pH 9, and at this pH the aneurin pyrophosphate molecule does not remain intact. It is of interest, however, that the distribution of the dephosphorylating activity for the phosphate bearing moiety is quite different from that for glycerophosphate.

The distribution of dephosphorylating activity for the different members of the second group of esters varies with each ester with two exceptions—those for the chalkone compounds are similar in some organs and those for stilboestrol and hexoestrol compounds are also similar but different from that for oestrone phosphate.

This work suggests that there are a number of stable specific dephosphorylating enzymes present in tissues which are different from the non-specific enzyme or enzymes which split glycero- and sugar phosphates and certain other phosphates.

The 'Anti-thrombic' Action of Heparin; The Relation of Sulphydryl Groups. By J. Green. (*North Western Group Laboratory, London, N.W.* 3)

Further study of the action of reducing agents on the 'anti-thrombic' action of heparin (Green, 1953) indicates that the findings can be grouped under three headings.

(1) Proportionate retardation of normal and heparinized plasma clotting.

Most reducing agents have this effect when incorporated in plasma. The increase in the apparent thrombin 'bound' by heparin is of the same order as the retardation of normal plasma. Exceptions to this rule, e.g. BAL, may be due to spontaneous oxidation of the reagent, but this is difficult to prove.

(2) True selective retardation of heparinized clotting.

All reducing agents tested have produced this effect when added to thrombin solutions, in concentrations below that at which normal plasma clotting is significantly retarded.

(3) Bizarre anti-heparin effects may develop, particularly when plasma containing BAL, or thrombin solutions with thioglycollate, dithionite or thiol-benzoate are left for a period of time; the stoicheiometric relation between heparin and 'thrombin' no longer obtains. Failure to reproduce this unique anti-heparin effect by oxidation of thioglycollate with H_2O_2 or alloxan, and in air or thrombin solutions, led to the view that it was due to reduction of 'thrombin' (Green, 1953). Incubation of the thioglycollate-thrombin under nitrogen, however, prevents the development of the anti-heparin effect, and on this, and other evidence it must be assumed to be associated with an oxidized form of the above SH reagents. There is a heat-stable factor in commercial thrombin which accelerates the aerobic oxidation of BAL.

The above findings are compatible with the hypothesis that the 'anti-thrombic' action of heparin is due to an effect on a preceding reaction in plasma clotting involving the formation of —S—S bonds, and not on the thrombin-fibrinogen reaction. This is in accord with recent views on plasma clotting (Lorand, 1950; Laki, 1953). Some results with *p*-chloro-mercuribenzoate lend support to this view.

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Taurine in Fresh and Iced Skeletal Muscle of Codling (*Gadus callarias*). By N. R. JONES.
(*Torry Research Station, Department of Scientific and Industrial Research, Aberdeen*)

Ethanol (75% v/v) extracts of codling muscle have been chloroform-separated (Awapara, 1948) and subjected to two-dimensional chromatography in phenol-ammonia and collidine-lutidine (Dent, 1948). Chromatograms showed a large ninhydrin-reducing spot, R_F values: phenol, 0·41; collidine, 0·40. This was not attributable to any basic extractive found in codling or the related gadoid haddock (*Gadus aeglefinus*) by separation on sulphonated polystyrene (SPX) columns (Shewan, 1953; Shewan, Fletcher, Partridge & Brimley, 1952).

Pretreatment of chromatograms with $CuCO_3.Cu(OH)_2$ (Crumpler & Dent, 1949) did not affect the running of the spot, and its detection in effluents from SPX columns indicated an acidic nature. R_F values in different solvents, final isolation from effluents by the method of Campbell & Work (1952), and also direct separation on Dowex 2 columns identified the substance as taurine. Estimated by a spot-elution method (Thompson, 1951) at 400–450 mg./100 g. muscle, taurine is quantitatively among the more important of the nitrogenous extractives. It has been reported in the liver and bile of other fish species (Ackermann & Mohr, 1937; Sihn and his collaborators: Asikari, Kim & Sihn, 1938; Sihn & Maeda, 1938; Sihn & Kim, 1938; Minato & Nisaburo, 1949) in herring (*Clupea harengus*) extractives (Mörner, 1937), and in dried Japanese cod (*Gadus Brandtii*) by Yoshimura & Kanai (1913).

Gutted codling, iced to simulate practical fish-trade conditions, became rapidly depleted of taurine. Some 40 mg./100 g. only remained after 21 days, the greatest rate of loss being over the first 4 days. Non-protein N fell from 380 to 260 mg./100 g. over 21 days.

Incubation of muscle minces and aerated mixed muscle and skin homogenates at 0° led to little change in taurine content over 6 days, although a heavy, typical marine spoilage microflora became established. It appears that the initial rapid drop in taurine in iced muscle probably derives from leaching by ice water.

This work was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Phosphorylation of Pantothenic Acid by *Lactobacillus arabinosus* 17–5. By J. BADDILEY, D. E. HUGHES, A. P. MATHIAS and W. S. PIERPOINT. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield and Lister Institute, London, S.E.* 1)

In previous papers it was shown that washed suspensions of *Lactobacillus arabinosus* 17–5 would synthesize coenzyme A (CoA) from pantothenic acid only if cystine were added; in the absence of cystine the pantothenic acid was taken up and bound by the cells as a phosphorylated derivative. The phosphorylated bound form was not available for the growth of *Lb. arabinosus*, but pantothenic acid was released quantitatively on treatment with alkaline phosphatase. In the present paper some further studies on the phosphorylation of pantothenate are reported.

In experiments with washed suspensions of intact cells (400 mg. dry wt.) the added pantothenic acid (500 μm-mole) was phosphorylated almost completely in 1 hr. The phosphorylated material was extracted from the cells and examined by paper chromatography and paper electrophoresis, and its behaviour compared with synthetic pantothenic acid 4′-phosphate, pantothenic acid 2′-phosphate, pantetheine and pantetheine 4′-phosphate. In three solvents (*iso*butyric acid/ammonium *iso*-butyrate/water; butanol/acetic acid/water; propanol/ammonium sulphate/water) spots were ob-

tained which contained the bulk of the phosphorylated pantothenate added and whose R_F's corresponded closely to that of pantothenic acid 4′-phosphate. The spots contained no detectable SH groups, but did contain material absorbing strongly at 260 mμ. This absorbing material was separated from the pantothenate by paper electrophoresis in sodium acetate or ammonium formate buffers.

Cells dried over P_2O_5, and extracts of cells broken by crushing at $-25°$ in a bacterial press (Hughes, 1951) would phosphorylate pantothenic acid, only if ATP was present. The amount of phosphorylation was increased by the addition of sodium fluoride (5×10^{-2}M). The stability in acid and alkali of the phosphorylated derivative prepared by dried cells corresponds to that of pantothenic acid 4′-phosphate.

These results suggest that pantothenic acid 4′-phosphate may be formed from pantothenic acid by *Lb. arabinosus*, prior to pantetheine formation. They also lend support to the suggestion of Brown & Snell (1953) that the product formed by incubating dried cells of *Proteus morganii* with ATP, pantothenic acid and cystine might be pantothenyl cysteine-4′ phosphate.

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Synthesis of Fatty Acids from Acetate by Preparation of the 'Cell-sap' of Rat Mammary Gland.

By G. Popják* and Alisa Tietz.† (*The National Institute for Medical Research, Mill Hill, London*)

The synthesis of short- and long-chain fatty acids from acetate by cell-free homogenates (suspensions of cell fragments) of rat and sheep mammary gland has previously been reported (Popják & Tietz, 1953*a*). High-speed centrifugal fractionation (at 25000 *g* and 104000 *g*) of homogenates of rat mammary gland yielded a soluble enzyme system, which is essentially the 'cell sap' of mammary gland cells diluted with buffer and which, after suitable additions, synthesizes short- and long-chain fatty acids from [^{14}C]acetate more efficiently than the original homogenates (Popják & Tietz, 1953*b*). The soluble preparations retained their activity after being kept frozen in solid CO_2 for several days. Whereas in the homogenates the addition of either pyruvate, oxaloacetate or α-oxoglutarate and of ATP was necessary to activate fatty acid synthesis from acetate, in the soluble enzyme system the addition of ATP (0·01M) alone was sufficient for activation. Nevertheless, oxaloacetate and α-oxoglutarate caused even in the soluble preparation a very significant stimulation of fatty acid synthesis. Without added ATP only traces of acetate were incorporated into fatty acids; the optimum concentration of both ATP and of acetate was 0·01M. The maximum synthesis of fatty acids by the soluble preparations was attained in 70 min. and 50% synthesis in 20 min. Incubation under N_2 caused, as with the homogenates, 80–90% inhibition. Among the various substances tested (2:4-dinitrophenol, 2×10^{-4}M; 2:6-dichlorophenolindophenol, 30 μg./ml.; KF, 0·05M; NaN_3, 0·01M; KCN, 0·01M; Na_3AsO_4, 0·05M; $HgCl_2$, 1×10^{-4}M; malonate, 0·05M) arsenate and Hg^{2+} caused 95%, and CN 70% inhibition of synthesis; 2:4-dinitrophenol was also slightly inhibitory. By chromatographic separation of the short- and long-chain fatty acids it was found that after 1 hr. incubation, three times as much ^{14}C was incorporated into the short-chain fatty acids (C_4–C_{10}) as into the long-chain acids (C_{12}–C_{18}).

The preparations of 'cell-sap' took up O_2, and it was found that the oxygen uptake was dependent on the presence of ATP, the highest $-Q_{O_2}$ being observed in the presence of 0·005M-ATP. Although the addition of substrates, especially of oxaloacetate or of α-oxoglutarate, stimulated O_2 uptake, nevertheless it appeared from experiments in which 2:6-dichlorophenol indophenol or NaN_3 was added to the preparations, that the cytochrome system is not involved in this oxygen uptake although cytochrome *c* is present in the enzyme preparations.

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The Biosynthesis of Riboflavin in *Eremothecium ashbyii*. By T. W. GOODWIN and S. PENDLINGTON. (*Department of Biochemistry, University of Liverpool*)

It is well known that *Eremothecium ashbyii* synthesizes large amounts of riboflavin, most of which is excreted into the culture medium; little is known, however, of the mechanism of synthesis. Using as basal medium containing (% w/v) glucose 1·0; $CaCl_2$, 0·02; $MgSO_4$, $7H_2O$, 0·1; NaCl, 0·02 and KH_2PO_4, 0·2, good growth and riboflavin production can be obtained by the addition of peptone, maximal concentration of riboflavin being achieved with 0·02 % (w/v) peptone N (compare Schopfer, 1944) and maximal yield with 0·04 %. Under these conditions growth is complete within 5 days, but riboflavin continues to be produced up to 8 days after inoculation.

The effect of amino acids on flavogenesis was examined by adding the amino acid under test at a level of 0·020 % N to the basal medium containing 0·005 % peptone N and comparing the results with those obtained using the basal medium containing 0·025 % peptone N. The amino acids fell into three groups: (*a*) those that had no effect on either growth or riboflavin synthesis—most of those tested; (*b*) those that stimulated both growth and flavogenesis equally—asparagine, aspartic acid, glutamic acid; and (*c*) those which stimulated riboflavin synthesis considerably without having any effect on growth—serine, threonine and (to a lesser extent) tyrosine. The optimum concentration of threonine N for riboflavin stimulation was eventually found to be 0·001 %; the D-isomer was inactive.

Various purines and pyrimidines were tested under the same conditions, and it was found that whilst adenine and xanthine (and adenosine) strongly stimulated riboflavin synthesis without affecting growth, the pyrimidines were in general without effect on either growth or flavogenesis. High levels of uracil slightly inhibited riboflavin synthesis. These observations confirm those of Maclaren (1952) made under different conditions.

The stimulatory effects of threonine, serine and tyrosine are not cumulative, whereas those of a purine plus one of these amino acids are cumulative.

A tentative conclusion from these results is that the amino acids provide no N for the biosynthesis of riboflavin but do provide C units for the synthesis of its aromatic ring, whilst the purines provide the remainder of the *iso*alloxazine structure. These ideas are being investigated using isotopes.

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The Arylsulphatase of an *Alcaligenes* Isolated from a Marine Mud. By K. S. DODGSON, T. H. MELVILLE, B. SPENCER and K. WILLIAMS. (*Physiology Institute and Department of Botany, University of Wales, Cardiff*)

Karunairatnam & Levvy (1951) showed the β-glucuronidase activity of sheep rumen to be due to bacteria. It was possible that the high arylsulphatase activity of marine molluscs (Dodgson, Lewis & Spencer, 1953) was of similar origin. Bacteria isolated from homogenates of the visceral humps of a number of molluscs showed no arylsulphatase activity when grown under varying conditions on plates containing 0·001M tri-potassium phenolphthalein disulphate (cf. Barber, Brooksbank & Kuper, 1951). Certain agar-digesting bacteria isolated from the red seaweed *Corallina* and other organisms isolated from several intertidal muds had no arylsulphatase. However, two micro-organisms present in intertidal mud collected at Sully, Glamorgan, showed considerable enzyme activity. One of these organisms was identified as a yeast, *Trichosporon cutaneum* and the other as a bacterium of the genus *Alcaligenes*. These arylsulphatase producers appeared to be localized in this particular area, since they were not found in mud samples taken from other parts of the Bristol Channel. Greatest activity was found in *Alcaligenes*, two-thirds of the total activity of the culture being associated with the bacterial mass. No increase in enzyme activity occurred when the organism was grown in the presence of potassium *p*-acetylphenyl sulphate.

Acetone-dried concentrates of the bacterial mass were maximally active at pH 8·75 in 0·1M phosphate when the concentration of potassium *p*-acetylphenyl sulphate was 0·003M, the enzyme being inhibited by excess of substrate (contrast the findings for the arylsulphatase of *Myco. piscium*; Whitehead, Morrison & Young, 1952). The high optimum pH was not an artifact arising from the acetone

treatment since an aqueous extract of the crushed bacteria behaved similarly. When potassium *p*-nitrophenyl sulphate (0·006M in 0·1M phosphate) was the substrate the optimum pH was also 8·75. The high optimum pH of this enzyme is interesting in view of the ability of *Alcaligenes* to produce alkali. During cultivation the pH of the medium increased from 7·5 to 8·7.

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A Method for the Isolation of Urinary Mucoproteins. By A. J. ANDERSON. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

At the moment two methods are available for the isolation of urinary mucoproteins. Both are precipitation reactions, one with 0·58M sodium chloride (Tamm & Horsfall, 1952) and the other with ethanol (Tamm & Horsfall, 1950). Both methods, however, suffer from certain disadvantages. The yield is low with the first method while with the second method the large amount of ethanol needed is a drawback. The following method relies on the adsorption of the mucoproteins on benzoic acid.

A 20% (w/v) acetone solution was added to normal male urine and the precipitate obtained treated with acetone, the residue remaining being treated with water at pH 9–9·5. After exhaustive dialysis the mucoprotein was precipitated by the addition of sodium chloride to 0·2M, the solution then being poured into five volumes of chilled acetone. A white powder (mucoprotein A) was obtained: yield 2·17 g. from 56 l. of urine (3·87 mg./100 ml. urine). Ethanol precipitation of the supernatant yielded a brown powder (mucoprotein B). Yield 256 mg. from 8·25 l. of urine (3·11 mg./100 ml. urine).

Both mucoproteins were readily water soluble and neither could be precipitated by the usual protein precipitants. Both gave a positive Molisch reaction. Mucoprotein A gave a positive biuret, ninhydrin, xanthoproteic, Millon and Sakaguchi reaction. Mucoprotein B gave a negative biuret and ninhydrin, the other reactions being either negative or very weakly positive. The Selivanoff and Bial tests for ketosugars and pentoses, respectively, were negative, as was the aldehyde test for tryptophan. Analysis (mucoprotein A and B, respectively): N, 10·54, 5·10%; acetyl (Kuhn & Roth, 1933), 3·67, 9·03%; hexosamine (Tracey, 1952), 14·41, 18·29%; reducing power expressed as glucose (before hydrolysis), 7·2, 3·6%; reducing power (the maximum liberated after hydrolysis with 2N-HCl at 100°), 17·5, 30·7%. The Morgan & Elson (1934) test for *N*-acetylhexosamine was positive for both proteins. It was also positive for the diffusate obtained on dialysis of the protein hydrolysates.

By the usual methods of paper chromatography (Consden, Gordon & Martin, 1944) the following amino acids were identified for both mucoproteins: aspartic acid, glutamic acid, phenylalanine, tyrosine, valine, proline, arginine, lysine, alanine, threonine, glycine, serine, leucine (*iso*leucine). Galactose, mannose and fucose were identified (Jermyn & Isherwood, 1949) and also glucosamine (Partridge, 1948). A second hexosamine, probably galactosamine, was present.

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Enzymic 11-β-Hydroxylation of Progesterone by Ox Adrenocortical Mitochondria. By A. C. BROWNIE and J. K. GRANT. (*Biochemistry Department, University of Edinburgh*)

It has been suggested (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker & Pincus, 1951) that progesterone is a key intermediate in the biosynthesis of the adrenocortical hormones. The determination of the course of metabolism of this steroid in the adrenal gland would thus appear to be of importance. Perfusion of progesterone through ox adrenals gave a variety of hydroxylated derivatives, of which 11-β-hydroxyprogesterone was obtained in less than 1% yield (Hechter *et al.* 1951). Incubation

of progesterone and of 17-α-hydroxyprogesterone with whole adrenal homogenates gave corticosterone and 17-α-hydroxycorticosterone respectively, but no 11-β-hydroxyprogesterone (Hayano & Dorfman, 1952). With washed adrenal homogenate residues, progesterone and 17-α-hydroxyprogesterone were inactive as substrates (Hayano & Dorfman, 1953). This observation led to the suggestion by these authors that 11-β-hydroxylation does not occur in the absence of a ^{21}C hydroxyl.

In the present work progesterone (45 mg.) was incubated aerobically for 1 hr. with ox adrenocortical mitochondria (400 mg. wet wt. mitochondria per 0·5 mg. progesterone) in a medium containing phosphate buffer (pH 7·4, 0·04 M), Mg^{2+} (0·005 M), KATP (0·0005 M) and K succinate (0·01 M). Progesterone was determined by absorption at 240 mμ., following recovery from incubation mixtures in 95 ± 3% yield, by solvent partition procedures. About half of the progesterone incubated was metabolized. From the products 8 mg. crystalline 11-β-hydroxyprogesterone was isolated by procedures involving seven-stage solvent distribution with double withdrawal (Craig & Craig, 1950) followed by chromatography on alumina and recrystallization. The product was identified by m.p. and mixed m.p. with authentic 11-β-hydroxyprogesterone, absorption spectra of a solution in sulphuric acid and by i.r. spectra.

As in the case of DOC (Brownie, Grant & Taylor, 1953) 11-β-hydroxylation of progesterone requires the concurrent oxidation of Krebs cycle acids. However, on account of the greater inhibition of the operation of the Krebs cycle in adrenocortical mitochondria by progesterone as compared with DOC 11-β-hydroxylation of progesterone could only be achieved with a suitably increased enzyme/steroid ratio.

These results show that preliminary 21-hydroxylation of progesterone is not necessary for 11-β-hydroxylation.

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Liver Catalase Activity in the Riboflavin Deficient Mouse. By D. H. Adams. (*Cancer Research Department, London Hospital Medical College, London, E.* 1)

There is a sex difference in mouse-liver catalase activity, males having the higher level. Castration of males results in a fall in enzyme activity, and the injection of testosterone into castrated males or normal females elevates their catalase activity to the normal male level. Adrenalectomy of normal females or castrated males produces a fall in catalase activity which is reversed by the injection of cortisone. Testosterone and cortisone appear to act independently on catalase (Adams, 1952).

The effect of riboflavin deficiency is now being studied in an attempt to gain more information about the normal mechanisms controlling liver catalase activity. Mice have been kept on a riboflavin-free diet, and catalase activity determined at intervals. As always in experiments of this nature two separate effects are involved: first, riboflavin deficiency, and secondly, the associated fall in total food intake. When weanling male mice are placed on a riboflavin-free diet, catalase levels tend to fall in both the deficient group and the pair-fed group receiving riboflavin, instead of rising to the higher adult level. This may be due to an interference with testicular development due to the lowering of food intake. If, however, the mice are injected with testosterone after 4–5 weeks on the diets, a rise in liver catalase activity to the normal adult level is obtained in the pair-fed controls, but not in the riboflavin deficient group. The dietary intake of weanling female mice kept under the same conditions is somewhat higher than in the males, and female catalase level remains normal in riboflavin-deficient, pair-fed, and *ad lib.* normal diet groups. Again, however, the catalase activity of the riboflavin deficient animals is not raised by testosterone, while the other groups react normally. In males continuously injected with testosterone after being kept for 3 weeks on the diets, catalase level rises in pair-fed groups and continues to fall in riboflavin deficient groups. If riboflavin is then given to the deficient animals (testosterone still being injected, and total dietary intake being held at the same level), their catalase activity rises to normal.

It appears therefore that testosterone will not affect catalase level in the riboflavin deficient mouse. The normal catalase level in riboflavin deficient females suggests that cortisone does not require the presence of riboflavin for its action on catalase.

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DEMONSTRATIONS

Porphyrins in the Urine of Normal Men. By A. COMFORT and M. WEATHERALL. (*Department of Pharmacology, London Hospital Medical College, London, E.* 1)

A Histochemical Study of the Specificity of Phosphatases. By G. H. BOURNE (introduced by F. L. WARREN). (*Department of Histology, London Hospital Medical College, London, E.* 1)

Light Scattering Apparatus By R. A. GRANT. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

A relatively simple apparatus has been developed for the determination of weight-average molecular weights of proteins and for use in kinetic studies of macromolecular systems.

Paper Ionophoresis of Phosphoric Esters. By M. W. NEIL and D. G. WALKER. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

Turba & Enenkel (1951) separated adenosine and its phosphates by paper electrophoresis in acetate buffer at pH 4. Schild & Maurer (1951) prepared a haemolysate of human red blood cells, which had been incubated with a saline solution containing radioactive phosphate ions. Small amounts of the preparation were placed on filter paper strips which were then wetted with veronal buffer, pH 8·6. After applying a p.d. across the ends of the strips for a period of time, they found that the distribution of radioactivity along the paper was concentrated in certain regions.

The technique of paper ionophoresis has now been applied in particular to the problem of separating mixtures of inorganic phosphates and triose and hexose phosphates. Phosphoric acid esters exist as anions in solution and move towards the anode in a variety of buffer solutions and over a wide range of pH values when a p.d. is applied across the ends of a strip of filter paper, wetted with buffer solution, on which the esters have been placed. Differences of pH with one particular buffer, and different buffers at the same pH, affect the rate of flow of the esters to a marked extent. Complete separations of all the esters have not been achieved, e.g. the hexose monophosphates run at almost identical rates under all the conditions tested. However, the ionophoresis technique has an advantage over paper chromatography (Walker & Warren, 1951) in that useful fractionations of tissue extracts may be achieved directly without preliminary treatment. The more classical methods and enzymic methods may then be applied to provide further more detailed analytical data. A scheme is proposed whereby such analyses may be undertaken.

Various methods for paper ionophoresis are demonstrated, together with some results obtained by such means.

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The Separation of Sugars and of Sugar Phosphates by Gradient Elution from Ion Exchange Columns. By C. W. PARR. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

Mixtures of monosaccharides, after adsorption as borate complexes on strong-base anion-exchange resins, have been separated and analysed by elution with weak borate solutions (Khym & Zill, 1951, 1952; Noggle & Zill, 1952). By eluting with mixtures of chloride and borate, hexose phosphates have more recently been resolved (Khym & Cohn, 1953).

The method to be demonstrated, while based on similar principles, was evolved independently of the work quoted above, and differs in many respects.

A fine grade of the strongly basic anion-exchange resin De-Acidite FF (Permutit Co. Ltd., London) is used and this is packed, resting on a sintered glass disk, in a glass column of internal cross-sectional area 1 cm.^{2}, so that the length of the resin bed is 10 cm. The resin is prepared in the borate form and mixtures of free sugars and hexose phosphates (up to a total of about 50 mg.) in aqueous solution are passed through the column and adsorbed.

Sucrose, fructose, glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate in the same mixture may be resolved by the following process of gradient elution: two identical reservoirs *A* and *B* (1 l. aspirator bottles) are kept at identical horizontal levels and are filled, *A* with 1 l. of 0·1 M boric acid adjusted to pH 7·0 with sodium hydroxide, and *B* with 1 l. of 0·1 M boric acid at pH 7·0 but containing also 0·25 M sodium chloride. The reservoirs are connected by a narrow tube so that, as boric acid flows from reservoir *A* (which is stirred electrically) into the column, so boric acid containing chloride flows from reservoir *B* to reservoir *A*, and the levels of liquid in *A* and *B* remain the same. With this apparatus the chloride concentration of the eluting solution, which is initially zero, increases linearly with the volume eluted, and becomes 0·25 M for a volume of 2 l. Portions (10 ml.) are collected, using an automatic fraction collector, and the various fractions are located using, in particular, the quantitative anthrone method described by Trevelyan & Harrison (1952).

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Photometry of Dyed Material in Filter Paper. By E. M. Crook, H. Harris, F. Hassan and F. L. Warren. (*Departments of Biochemistry, University College London and London Hospital Medical College*)

Genetics of Cystinuria. By H. Harris, Ursula Mittwoch, Elizabeth B. Robson and F. L. Warren. (*Galton Laboratory, University College London and Department of Biochemistry, London Hospital Medical College*)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **325th Meeting** *of the Biochemical Society was held in the Department of Chemical Pathology, The Postgraduate Medical School of London, Ducane Road, London, W. 12, on Saturday, January 16th, 1954, at* 11 a.m., *when the following papers were read:*

COMMUNICATIONS

The Activity of the Enzymes Sulphatase and Glucuronidase in the Urine of Normal and Cancer Patients. By E. Boyland, D. M. Wallace and D. C. Williams. (*Chester Beatty Research Institute, London, S.W.* 3)

Many facts suggest that cancer of the bladder in man is caused by carcinogenic substances present in urine. Some of the suspected carcinogens are excreted in 'detoxicated' or conjugated forms from which active carcinogens would be released in the bladder by the action of appropriate enzymes, such as sulphatase and glucuronidase.

The activities of urinary sulphatase and glucuronidase have been measured in groups of (*a*) 58 patients with cancer of the bladder, (*b*) 11 patients with tumours of other sites, (*c*) 6 patients suffering from renal tuberculosis, (*d*) 19 subjects free of malignant disease of which 5 had heavily infected urine. The concentration of glucuronidase and sulphatase was high in cases of bladder cancer. Only one case in this group (*a*) had values for glucuronidase lower than the highest value found in the group (*d*) of controls, and this was an extremely dilute urine, the 24 hr. specimen measuring 7·5 l. Twelve cases of the same group had sulphatase values lower than the highest value of the control group.

One patient with cancer of the larynx had abnormally high glucuronidase and sulphatase, in an abnormally small volume of urine (650 ml.) for the 24 hr. specimen, and one patient with cancer of the stomach and one with lymphatic leukaemia had raised glucuronidase. Values within the normal range for both enzymes were found among 9 patients with cancer at other sites. Glucuronidase was high in all of the patients in group (*c*) with renal tuberculosis under treatment with drugs. We do not know if the raised urinary enzyme activities are the cause of the bladder cancer but they remain high after removal of the tumours, and the glucuronidase appears to fall after treatment with stilboestrol (4:4′-dihydroxy-$\alpha\beta$-diethylstilbene). The activity of the enzymes is higher in bladder tumour tissue than in normal bladder tissue.

Carbohydrate Metabolism and Plasma Adrenaline Level. By H. Weil-Malherbe. (*Runwell Hospital, Wickford, Essex*)

It has previously been shown (Weil-Malherbe & Bone, 1952; Weil-Malherbe, 1953 *a*, *b*) that insulin-induced hypoglycaemia is preceded by a fall of the plasma adrenaline concentration and that, conversely, termination of hypoglycaemia, whether spontaneous or induced by glucose, is accompanied by a rise of the plasma adrenaline concentration to a level which temporarily exceeds the resting level. It has now been found that the ingestion of 50 g. glucose by a fasting human subject leads to an immediate and brief rise of the plasma adrenaline concentration which reaches its peak after 5–10 min. and returns to the starting value after 20–30 min. The maximum increase of concentration usually amounts to 50–100%; it is observed before the blood-sugar concentration has notably increased. There is no change of the noradrenaline concentration.

In marked contrast to the effect of glucose intake no significant changes in the plasma adrenaline level are found when 50 g. fructose are taken by a fasting subject.

It has been suggested that insulin administration causes an increased fixation of adrenaline by the tissues. To test this hypothesis arterio-venous differences were investigated. It was found that the fall of the adrenaline level in venous plasma precedes that in the arterial plasma; consequently there is a temporary increase of the adrenaline arterio-venous difference occurring within the first 5 min. after an intravenous injection of insulin.

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The Quantitative Determination of Bile Pigments in Serum using Reverse Phase Partition Chromatography. By B. H. Billing. (*Postgraduate Medical School, London, W.* 12)

Cole & Lathe (1953), using reverse phase chromatography, have separated the bile pigments from jaundiced serum into two groups, which can be distinguished by their different reaction in the Van den Bergh test. If a butanol/water (pH 6·0) system is employed (Cole, Lathe & Billing, 1953), then bilirubin and two direct-reacting pigments are obtained.

This procedure has been adapted so that a quantitative determination of the three pigments can be made. The results obtained for various clinical conditions will be discussed, together with those for the sera of rats, which have been made jaundiced by tying the bile duct or by the intravenous infusion of bilirubin. A reasonable correlation has been found for the percentage of direct-reacting pigments found chromatographically and that obtained by the Malloy & Evelyn (1937) colorimetric determination after 30 min.

The treatment of serum from cases of obstructive jaundice with freshly diazotized sulphanilic acid in the presence of ethanol gives two diazonium components, with similar absorption spectra in the visible region. These have been separated on reverse-phase partition columns of silicone-treated kieselguhr, using the system prepared from 50 vol. *n*-butanol, 45 vol. water and 5 vol. 0·1 M citrate buffer pH 4·0. With 1·5 g. silicone-treated kieselguhr and 0·75 ml. of the non-polar phase, the slow-moving component (pigment A) has an R value of 0·1 and the fast-moving component (pigment B) has an R value of 0·7. Bilirubin gives rise to pigment A while direct pigment I gives both pigments A and B, and direct pigment II gives pigment B.

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The Specificity of the Dinitrobenzene Reaction for Ketosteroids. By Irene E. Broadbent and W. Klyne. (*Postgraduate Medical School, London, W.* 12)

Reaction with dinitrobenzene and alkali in ethanol is employed almost universally for the determination of urinary 17-ketosteroids (oxosteroids). (See, for example, Zimmermann, 1935, 1936, 1944; Callow, Callow & Emmens, 1938; Medical Research Council Committee on Clinical Endocrinology, 1951.) Some studies on the specificity of this reaction, which is really a reaction of activated methylene groups, have been made in the past (see the references above and also Kaziro & Shimada, 1937; Hansen, Cantarow, Rakoff & Paschkis, 1943). Studies have now been made using a wider range of steroid ketones than has been available previously, and also some triterpenoid ketones. The Medical Research Council (1951) method has been followed, and parallel experiments have been carried out with colour development at 25° for 5 min. as well as for 60 min. The principal results so far obtained are as follows; values for 10^{-3} ϵ are given in parentheses.

A high extinction at 520 mμ. (15·2 at 60 min.) seems to be highly specific for steroid 17-ketones. A 17-ketone derived from lanosterol (Barnes, Barton, Cole, Fawcett & Thomas, 1953) gives a peak in a similar position (540 mμ.), but its intensity (1·0) is only a very small fraction of that obtained with ordinary 17-oxosteroids.

An *A*-nor-3-oxosteroid (*A*/*B cis*) shows a very characteristic behaviour; a maximum at 510 mμ. (6·4) after 5 min., and a curve with high absorption from 300 to 400 mμ. and a peak at 410 mμ. (8·6) after 60 min. Two other five-membered ring ketones, an *A*-nor-2-ketone and a 15-ketone (Barnes, Barton & Laws, 1953) show a maximum at about 400 mμ. (2·2–2·6) after 60 min.

3-Ketones of the 5α-steroid (*A*/*B trans*) series give a broad maximum at 540 mμ. (6·1–6·8) after 5 min., but after 60 min. this peak has disappeared and the absorption curve falls continuously from 300 to 600 mμ. 3-Ketones of the 5β-steroid series (*A*/*B cis*) give a small peak at 360 mμ. (approx. 6) at 5 min.; after 60 min. the spectra resemble those of the *A*/*B trans* compounds. These facts may be useful in structural studies. 3-Oxotriterpenoids (e.g. olean-12-en-3-one, 'β-amyrone') show at 5 min. absorption curves rather similar to that of a 3-oxo-5α-steroid; the triterpenoid curves do not change greatly after 60 min.

Cholestan-2-one shows at 5 min. a high maximum (11·0) at 540 mμ.; at 60 min. this peak is broader and lower (530 mμ., 7·2).

A 4-oxosteroid and a 17*a*-oxo-*D*-homosteroid show rather similar curves at 60 min., each with two peaks at about 320 and 420 mμ.; the maxima for the 4-ketone are much higher than those for the 17*a*-ketone. 6-, 7-, 11- and 12-ketones show practically no absorption (cf. Kaziro & Shimada, 1937).

Steroid 20-ketones (and also Δ^{16}-20-ketones) show a maximum at 490 mμ. (2·4) after 60 min. (cf. Callow *et al.* 1938; Hansen *et al.* 1943).

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Fluorescent Indicators of Adsorption in Aqueous Solution and on the Solid Phase. By G. Weber and D. J. R. Laurence. (*Department of Biochemistry, University of Sheffield and Postgraduate Medical School, London, W.* 12)

In the naphthalene and acridine series, the introduction of an anilino group or of certain substituted anilino groups results in a compound non-fluorescent in aqueous solution, but brightly fluorescent when adsorbed in solution or on the solid phase. Examples are 5(4′-carboxyanilino)2-chloro-7-methoxyacridine and the corresponding 4′-sulphonic and arsonic acids, the 1-anilinonaphthalene-5- or 8-sulphonic acids and the 2-anilinonaphthalene-6-sulphonic or -6:8-disulphonic acids. Similar compounds in which the anilino group is replaced by a benzylamino or a phenyl group are brightly fluorescent in aqueous solution, in contrast with the anilino-substituted compounds. Although the preparation of some of these substances has been described in detail, the increase in fluorescent efficiency with adsorption does not appear to have been recorded. The relation between molecular rigidity in solution and fluorescent efficiency has led to the conclusion (Forster, 1946; Hofer, Grabenstetter & Wiigo, 1950) that only planar molecules are fluorescent. The present observations afford a striking example of this rule. Solutions of the compounds in certain organic solvents are fluorescent, so that the quenching in aqueous solution appears to be related to solute-solvent interactions on which the polar character of the solvent has marked influence. Quenching due to dimer or micelle formation is excluded, since the crystals are fluorescent and the quenching is not reversed by rise in temperature.

The aqueous solutions of the compounds become brightly fluorescent following the addition of solutions of adsorbing proteins such as serum albumin or heat denatured proteins. Most native proteins, e.g. γ-globulin, Bence Jones protein, ovalbumin, do not adsorb the compounds or activate the fluorescence.

The increase in fluorescence with protein concentration corresponds quantitatively with changes in the absorption spectra. The extent of the interactions at various pH values, with or without the addition of competing substances, can be estimated directly from the fluorescent intensities.

The application of this group of substances to a variety of problems is discussed and is illustrated elsewhere at this meeting.

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The Dye-binding Capacity of Human Plasma Determined Fluorimetrically, and its Relation to the Determination of Plasma Albumin. By Joyce E. Fildes, D. J. R. Laurence and Valerie H. Rees. (*Postgraduate Medical School, London, W.* 12)

1-Anilinonaphthalene-8-sulphonic acid (A) is non-fluorescent in aqueous solution, but becomes brightly fluorescent when adsorbed on to protein molecules in solution (Weber & Laurence, 1954). Of the proteins of blood plasma, albumin has the specific property of adsorbing anions, and so the fluorescence of solutions containing blood plasma with an excess of A is a function (almost linear) of the albumin concentration.

The present communication gives comparisons made for pathological plasma between the albumin concentrations determined fluorimetrically and those determined by salt fractionation or by electrophoresis on filter paper. In the fluorimetry, plasma (0·05 ml.) was added to a solution (7 ml.) containing 4×10^{-5} g. of A buffered at pH 7·0. The fluorescence was excited by the 365 mμ. line of the Hg arc, and measured using a photomultiplier. Crystalline human albumin was used to calibrate the method and crystalline bovine albumin was used as a substandard. The salt fractionation was that of Campbell & Hanna (1937). The electrophoresis was as described by Knedel (1951). The dyed filter-paper strips were scanned by means of a densitometer, and

the optical density of the dye, without correction, was used as an estimate of relative protein concentrations along the paper. The absolute protein concentrations were obtained using the total protein concentration by the copper sulphate drop method of Van Slyke. An electrophoresis technique for which a standard albumin solution was run ahead of the plasma sample and the albumin fractions eluted and compared colorimetrically was also used.

Good agreement between the fluorimetric method and the electrophoretic methods was obtained if the automatic densitometer (Laurence, 1954) (S.D. = 0·17 g./100 ml.; 103 cases) or the elution technique (S.D. = 0·13 g./100 ml.; 28 cases) were used. Icteric plasma was an exception and possible reasons for the low results obtained with jaundiced samples are discussed.

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The Use of a Compound Forming Fluorescent Conjugates with Protein in Studying the Mode of Action of Polymyxin. By B. A. Newton. (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

The addition of polymyxin to washed cell suspensions of *Pseudomonas aeruginosa* results in the leakage of soluble constituents from the cells. It has been suggested that the bactericidal activity of this antibiotic may be due to its ability to combine with certain chemical groups at the cell surface with a resultant disorganization of a cell membrane or osmotic barrier (Newton, 1953*a*). Similar findings have been described for other organisms (Few & Schulman, 1953). Cells can be protected against the bactericidal activity of polymyxin by certain cations which compete with the antibiotic for sites on the cells (Newton, 1953*b*).

N-Tolylnaphthylamine-8-sulphonic acid (TNS) forms conjugates with protein which fluoresce when excited by ultraviolet light (Weber, 1954). If washed cell suspensions of *Ps. aeruginosa* are treated with TNS no fluorescence develops; if polymyxin is also added fluorescence develops rapidly, demonstrating that TNS can then penetrate to protein-containing portions of the cells. The rate of development of fluorescence is markedly decreased by the presence of certain cations which appear to compete with polymyxin for combining groups on the cells. From a comparison of the affinities of these cations for the polymyxin-combining groups of the cells with their ability to reverse the charge on certain colloids (Bungenburg de Jong, 1949), it is suggested that the polymyxin combining loci of the cells may be polyphosphates.

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Protein-bound Polysaccharides in Diabetes With and Without Complications. By I. C. Gilliland, M. G. Hanno and J. I. Strudwick (introduced by E. J. King). (*Postgraduate Medical School, London, W.* 12)

Histological studies of both the renal and retinal complications of diabetes show a peculiar hyalinization of the arterioles, which, since it stains with periodic acid-Schiff stain, contains protein-bound polysaccharides. It has been suggested that these lesions are the result of haematogenous deposits (McManus, 1950; Duguid & Anderson, 1952). Therefore the serum protein-bound polysaccharides have been studied in diabetics graded according to the severity of their complications.

Four groups were chosen: (1) normal healthy volunteers; (2) diabetics without complications; (3) diabetics with complications excluding kidney disease; (4) diabetics with Kimmelstiel-Wilson syndrome (Gilliland, 1951).

The serum was obtained from diabetics who were apparently controlled and had not recently been in coma or acidosis.

The total serum proteins were measured by specific gravity and their fractions by paper electrophoresis using napthalene-black staining. Total protein-bound polysaccharides were measured as the sum of protein-bound non-glucosamine carbohydrate by the anthrone reagent (Dreywood, 1946;

Graff, Greenspan, Lehman & Holechek, 1951) and protein-bound glucosamine by a modification of the Elson & Morgan (1933) procedure. Their relative amounts in protein fractions were determined by paper electrophoresis followed by PAS staining (Koïw & Grönwall, 1953). In both cases the stained papers were read by the recording densitometer (Laurence, 1953).

The results show that in normal healthy people the maximum protein-bound polysaccharide concentration occurs in the α_2 fraction.

There is a progressive rise in the total protein-bound polysaccharides as diabetic complications increase (Keiding, 1953), and considerable changes are demonstrable before the clinical kidney complications ensue. The increases shown by electrophoresis were highly significant in the α_2 fraction and significant in the β fraction. There was no statistical difference in the ratio of the two total carbohydrate fractions measured and no evidence of an abnormal protein-bound polysaccharide was found. The same order of difference could be discerned between the proteins as between the protein-bound polysaccharides by analysis of the electrophoretic pattern. The protein and protein-bound polysaccharide electrophoretic patterns are characteristic in Kimmelstiel-Wilson syndrome. A very similar pattern of protein and protein-bound polysaccharides as determined by electrophoresis occurs in the serum of patients with amyloid disease. These findings tend to support the suggestion that the protein-bound polysaccharides which occur in the pathological lesion may be haematogenous in origin.

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Silica and Collagen in the Lungs and Lymph Nodes of Cortisone-treated Silicotic Rats. By B. D. Stacy and E. J. King. (*Postgraduate Medical School, London, W.* 12)

The inhibiting effect of cortisone on proliferating connective tissue and phagocytosis led to observations on the effect of this hormone on the developing silicotic nodule. Short-term studies on the effect of cortisone on the reaction of the mouse peritoneum to quartz showed a delay in macrophage response (Curran, 1952) and an inhibition of fibrosis (Magarey & Gough, 1952; Schiller, 1953). Over a longer period the effect of cortisone upon fibrosis has been observed in rats injected intratracheally with quartz. In one experiment the effect of cortisone on developing silicosis was studied (Harrison, King, Dale & Sichel, 1952), the hormone treatment being commenced at the time of the quartz injection. In a second experiment designed to test the effect of cortisone on established silicosis, cortisone treatment was commenced 100 days after the quartz injection (King, Harrison & Attygalle, 1954).

This paper reports the results of a chemical examination of the lungs and the hilar lymph glands from animals used in these two experiments. The material was bulked into convenient groups, the lungs analysed for silica and collagen and the lymph glands for silica.

Silica was analysed by weighing the acid-in-soluble residue from the ash of an appropriate sample. Collagen was estimated by determining the hydroxyproline content of lung hydrolysates, the method of Neuman & Logan (1950*a*, *b*) being slightly modified to allow of the estimation of collagen in fixed tissue (Stacy, 1954).

From the chemical results it was clear that, both in developing and established silicosis, cortisone markedly decreased the transport of dust from the lungs to the lymph glands and to some extent inhibited the formation of collagen in the lungs. These results are in general agreement with histological findings concerning cortisone and developing silicosis.

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Removal of Nucleotide from B_{12} Vitamins. By DOROTHY E. GANT, E. LESTER SMITH and L. F. J. PARKER. (*Glaxo Laboratories, Ltd., Greenford, Middlesex*)

The acid hydrolysis of vitamin B_{12} can follow alternative paths. Cold dilute mineral acids slowly hydrolyse three labile amide groups to yield successively acids with one, two and three carboxyl groups. Seven acids, three isomeric mono-, three di- and one tri-basic, have been isolated in crystalline form by paper electrophoresis and chromatography. They have all been reconverted into vitamin B_{12} by treatment first with chloroformic ester and triethylamine in anhydrous dimethylformamide and then with ammonia. Concentrated hydrochloric acid at 65° for 5 min. removes the nucleotide with little attack on the amide linkages (Armitage, Cannon, Johnson, Parker, Lester Smith, Stafford & Todd, 1953). Perchloric acid (72%, w/v) gives a better yield of the main product, which behaves on paper electrophoresis as a neutral substance in its purple dicyan form but as a base in its orange form with no cyanide. In these respects, and also in chromatographic behaviour, it is indistinguishable from factor B isolated from calf manure and by fermentation with a *Streptomyces griseus* strain (Ford & Porter, 1952). Identity was confirmed by chromatographing, on paper with wet *sec.*-butanol, a mixture of fermentation factor B with 'denucleotided' radioactive (^{60}Co) vitamin B_{12}. Material eluted from the top, middle and bottom of the single zone showed the same specific activity; this test would discriminate between substances having R_F values so close that the zones overlapped. Moreover, like the natural factor B, the product from strong acid hydrolysis of vitamin B_{12} showed high microbiological activity (about three times that of vitamin B_{12}) by the *Escherichia coli* mutant plate assay, but much lower activity by the *E. coli* tube assay and no activity towards *Lactobacillus leichmannii*.

Factor A and pseudo vitamin B_{12} (Holdsworth, 1953 and next communication) have been treated similarly with concentrated HCl. The main coloured product from each behaved identically with factor B in microbiological assays; also on electrophoresis in acetic acid or in phosphate buffer at pH 6·5 and on paper chromatography. Moreover, chromatography of the crude reaction mixtures from these factors, alongside that from vitamin B_{12}, revealed the same complex pattern in all three; the minor zones arise from isomeric mono- and di-carboxylic acids lacking nucleotide. It may therefore be concluded that both factor A and pseudo vitamin B_{12} differ from vitamin B_{12} solely in the nature of the nucleotide components.

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New Purines in B_{12} Vitamins. By F. B. BROWN and E. LESTER SMITH. (*Glaxo Laboratories, Ltd., Greenford, Middlesex*)

We have been able to prepare a few mg. of chromatographically and electrophoretically pure pseudo vitamin B_{12} from 160 kg. of pig manure and have confirmed by paper chromatography in two solvent systems that it yields adenine on hydrolysis with boiling 2N-HCl, as reported by Dion, Calkins & Pfiffner (1952). On warming with sodium nitrite in dilute acetic acid, pseudo vitamin B_{12} is deaminated to an electrophoretically neutral, red, crystalline substance with absorption maxima at 359, 516 and 540 mμ. It is active towards the *Escherichia coli* mutant but only slightly towards *Lactobacillus leichmannii*. What appears to be the same substance has been isolated in traces from pig manure and tentatively named factor 'G'. On hydrolysis, factor 'G' and the deamination product both yield a substance chromatographically indistinguishable from hypoxanthine, the product to be expected if the original adenine moiety suffers deamination.

Pure factor 'A' (Ford & Porter, 1952) has been prepared in somewhat larger amounts from calf and pig manure. It also can be transformed from a basic to a crystalline neutral substance by treatment with nitrous acid. This deamination product has absorption maxima at 358·5, 517 and 540 mμ. It is active towards the *E. coli* mutant but only slightly towards *L. leichmannii*. A substance from pig manure tentatively named factor 'H' appears to be identical with it.

On hydrolysis with boiling 2N-HCl, factor 'A' and deaminated factor 'A' both yielded purines identified as such on paper chromatograms by dark zones in short-wave ultraviolet light and by the copper staining reagent. The R_F values in *n*-butanol with either ammonia, or ammonia plus diethyleneglycol, corresponded with those of 2-methyladenine and 2-methylhypoxanthine respectively. Factor 'H' similarly yielded 2-methylhypoxanthine. More-

over the absorption maxima of these synthetic purines in neutral, acid and alkaline solutions corresponded exactly with those of the hydrolysis products of factor 'A' and deaminated factor 'A' respectively. The identity of each pair was further established by their corresponding mobilities on paper electrophoresis in acetic acid.

It therefore seems permissible to announce the presence in two B_{12} vitamins, of the purines 2-methyladenine and 2-methylhypoxanthine, not previously found in nature.

We are grateful to Professor A. R. Todd, F.R.S., for samples of reference purines.

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Biosynthetic Relationships between Vitamin B_{12}-like Factors. By J. E. Ford and E. S. Holdsworth. (*National Institute for Research in Dairying, University of Reading*)

The demonstration that factor A, pseudo vitamin B_{12} and cyanocobalamin differ only in the constitution of the nucleotide portion of the molecule and that factor B is their common base (Gant, Smith & Parker, 1954) suggests an explanation of the occurrence of all these substances in the gut. It seems likely that the formation of a particular factor depends on the relative abundance of the right nucleotide or its precursor.

In fact we were able to guide the synthetic ability of a vitamin B_{12}-requiring mutant of *Escherichia coli*, which normally produces factor C from factor B (Ford, Kon & Porter, 1952), by adding to the medium in the presence of factor B the nucleotide of factor A, pseudo vitamin B_{12} and cyanocobalamin, when the corresponding vitamins were produced.

The effect of adding various purines, pyrimidines and vitamins was also investigated. Pseudo vitamin B_{12} was synthesized when adenine was added to the system, in accord with the finding that adenine is present in its nucleotide (Dion, Calkins & Pfiffner, 1952). Adenine could not be replaced by adenosine, adenylic acid or ATP, suggesting that the sugar in the nucleotide resembles that in cyanocobalamin and is not the β-form.

Until the demonstration of the presence of 2-methyladenine in factor A (Smith, 1954) our search for the base of the nucleotide of this substance among common purines and pyrimidines was unsuccessful.

We have observed that with riboflavin, cyanocobalamin is produced. More efficient 'shunting' of the synthesis towards cyanocobalamin took place when various parts of the riboflavin molecule and related substances were tested, e.g. 1-amino-3:4-dimethyl-6-D-ribityl-aminobenzene, 4:5-dimethyl-*o*-phenylenediamine, and 5:6-dimethylbenziminazole all lead to the production of cyanocobalamin. This finding is further experimental evidence for Woolley's view that dimethyl-*o*-phenylenediamine is a common precursor of cyanocobalamin and riboflavin.

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Observations on the Molecular Weight of Human Fibrinogen. By E. A. Caspary and R. A. Kekwick. (*The Lister Institute, London, S.W. 3*)

The examination of highly purified human fibrinogen by sedimentation, diffusion, and electrophoresis has provided data suggesting that in dilute solution the fibrinogen molecule disassociates.

The fibrinogen preparations studied were obtained by fractionation in systems containing ethyl ether (Kekwick, Mackay, Nance & Record, unpublished). Of the protein nitrogen 97–98 % was recovered in the clot formed with thrombin, and the preparations were essentially free from plasminogen and plasmin.

After prolonged electrophoresis (300 mA/hr.) in phosphate buffer, pH 8, $I = 0{\cdot}2$, the ascending (anode) boundary displayed a single peak, but the configuration of the descending (cathode) boundary was complex.

In the ultracentrifuge in phosphate pH 8·0, $I = 0{\cdot}2$ + NaCl, $I = 0{\cdot}15$, a single peak was obtained. The sedimentation coefficient increased linearly as the concentration was reduced but fell sharply below 0·15 g. fibrinogen/100 ml., at which point there was a corresponding marked increase in diffusion coefficient.

Linear extrapolation to infinite dilution of measurements over the concentration range 0·2–1·0 g./100 ml. gives $S_{20\,w}$ 7·63 ± 0·07 (S.E.) × 10^{-13} and $D_{20\,w}$ 1·97 ± 0·06 (S.E.) × 10^{-7} cm.²/sec. Taking 0·725 for the partial specific volume these give a molecular weight of 341 000 ± 10 000. The apparent molecular weight calculated from the diffusion coefficient at 0·05 g./100 ml. and a corresponding extrapolated sedimentation coefficient is 130 000 ± 20 000. The frictional coefficients are 2·34 and 1·33 respectively, leading to axial ratios of 28 and 6, assuming no hydration.

Oncley, Scatchard & Brown (1947) obtained a molecular weight of 400 000 for human fibrinogen from sedimentation and viscosity measurements, and 580 000 by osmotic pressure. From recent light-scattering measurements on bovine fibrinogen (Katz, Gutfreund, Shulman & Ferry (1952)) give 340 000 for the molecular weight.

The measurements reported here suggest that previous values for the molecular weight of human fibrinogen may be too high. It is of interest that a disassociation of the fibrinogen molecule appears to occur at a concentration close to that at which fibrinogen is present in human plasma.

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Studies on Aconitase. By J. F. Morrison. (*Department of Biochemistry, University of Oxford*)

The highly purified aconitase preparation of Morrison (1954) provided a means for studying the relations between the enzyme activity and the concentration of Fe^{2+} and reducing agents (Dickman & Cloutier, 1951; Morrison, 1953). The observations on the relationship between the Fe^{2+} concentration and the reaction velocity with cysteine or ascorbic acid conformed to a simple Michaelis-Menten relation and gave apparent dissociation constants for the Fe^{2+}-enzyme complex of $3·9 \times 10^{-6}$ M (cysteine) and $1·7 \times 10^{-5}$ M (ascorbic acid). A simple Michaelis-Menten relation was also found between the enzyme activity and the concentration of cysteine, ascorbic acid and thioglycollate in the presence of Fe^{2+}. The apparent dissociation constants of the enzyme-reducing agent complexes were $3·6 \times 10^{-3}$ M (cysteine), $1·2 \times 10^{-3}$ M (ascorbic acid) and $6·0 \times 10^{-3}$ M (thioglycollate). The results are consistent with the idea that one Fe^{2+} ion and one molecule of reducing agent combine with each active centre of aconitase to form the active enzyme complex which is suggested to be either an enzyme-Fe^{2+}-reducing agent or an enzyme-Fe^{2+}-activator complex. The behaviour of glutathione was anomalous.

The pH optimum of the reactions *iso*citrate → *cis*-aconitate, *cis*-aconitate → citrate and *iso*citrate → citrate was the same in the presence of a particular buffer and altered to the same extent in different buffers. The following pH optima were found: glycerophosphate 7·5, phosphate 7·7, *N*-ethylmorpholine 8·1, and veronal-acetate 8·6.

The finding of Krebs & Holzach (1952) that there was a definite lag period in the conversion of citrate → *iso*citrate was confirmed. A lag period was also found for the reverse reaction. The Michaelis constants of aconitase were found to be $3·6 \times 10^{-3}$ M for citrate, $1·2 \times 10^{-4}$ M for *cis*-aconitate and $4·8 \times 10^{-4}$ M for D-*iso*citrate in 0·05 M phosphate buffer at pH 7·7 and 22° when the enzyme was activated with Fe^{2+} and cysteine. The results obtained from a study of the reaction kinetics suggested that one catalytic centre of the aconitase protein was concerned in the reactions catalysed by aconitase.

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The Inhibition of Aconitase by Fluorocitrate. By J. F. Morrison and R. A. Peters. (*Department of Biochemistry, University of Oxford*)

Enzymically prepared fluorocitrate (Peters, Wakelin, Buffa & Thomas, 1953*a*) is a competitive inhibitor of the highly purified aconitase preparation described by Morrison (1954). This proves the preliminary results of Peters & Wilson (1952). The apparent dissociation constant of the aconitase-fluorocitrate complex is $8·7 \times 10^{-5}$ M; the inhibition can be reversed by increasing the substrate/inhibitor ratio.

Synthetic fluorocitrate prepared by Rivett (1953) behaves differently. It is far more toxic to the soluble aconitase. When the initial reaction velo-

cities in the presence and absence of fluorocitrate are plotted according to Lineweaver & Burk (1934), a graph characteristic of competitive inhibition is obtained. The apparent dissociation constant of the aconitase-fluorocitrate complex is $8{\cdot}4 \times 10^{-6}$M with D-*iso*citrate and 12×10^{-6}M with citrate as substrates. Reversal of the inhibition of aconitase by synthetic fluorocitrate with increasing substrate concentrations can be observed, but the inhibition by higher concentrations of fluorocitrate has not been completely reversed. There is some indication that an irreversible inhibition may also occur, especially when the enzyme is pre-incubated with synthetic fluorocitrate.

It will be noted that this effect on soluble aconitase is the reverse of that shown to occur with particle preparations (Peters, Wakelin, Rivett & Thomas, 1953*b*). It seems as though the greater toxicity of synthetic fluorocitrate is connected with the presence of 'unnatural' stereoisomers which would not be expected to be present in the enzymically prepared fluorocitrate.

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pH Changes Accompanying the Formation of Metmyoglobin Complexes. By P. George and G. I. H. Hanania. (*Department of Colloid Science, University of Cambridge*)

If in the fluoride and cyanide complexes of metmyoglobin the anions and not their conjugate acids are bonded to the haematin iron atom, $Fe^+(H_2O)$, then the simplest equilibria which could be set up in solutions about pH 7 are

$$Fe^+(H_2O) + F^- = FeF + H_2O, \quad \text{(i)}$$

$$Fe^+(H_2O) + HCN = FeCN + H_3O^+. \quad \text{(ii)}$$

In solutions about pH 10, which are alkaline with respect to the ionization of HCN (pK = 9·4) and of the iron-bound H_2O molecule (pK = 9·0), the corresponding equilibria are

$$FeOH + H^+ + F^- = FeF + H_2O, \quad \text{(iii)}$$

$$FeOH + H^+ + CN^- = FeCN + H_2O. \quad \text{(iv)}$$

Direct measurements have been made of the pH changes which accompany the formation of the fluoride and cyanide complexes in unbuffered solutions at initial pH values of about 7·0 and 10·0, and the results were found to be as indicated in the above equations namely: no change in (i), one equivalent H^+ formed in (ii), one equivalent used up in each of (iii) and (iv). These results appear to rule out the possibility of the bonding of HF or HCN in these complexes.

However, it is also possible to account for the observed pH changes even though the bonding of the acids HF and HCN in these complexes is assumed, provided that the ionization of a haem-linked acidic group in metmyoglobin is superimposed on the changes indicated in the above equations. That is, if a haem-linked group $\overset{+}{H}Y$ which is not ionized in metmyoglobin at pH 7·0 becomes completely dissociated in the complex, then equations (i) and (ii) may be rewritten in the form

$$\overset{+}{H}Y.Fe^+(H_2O) + F^- = Y.Fe(HF) + H_2O, \quad \text{(v)}$$

$$\overset{+}{H}Y.\overset{+}{F}e(H_2O) + HCN = Y.Fe(HCN) + H_3O^+, \quad \text{(vi)}$$

and similarly for equations (iii) and (iv). It may therefore appear impossible to distinguish between the two modes of combination in these complexes.

This type of difficulty can be resolved if pK values for linked groups in myoglobin derivatives are obtained from studies of reactions where no changes in hydrogen ion concentration otherwise occur, for instance the oxygenation and electrode reactions. Analysis of the data obtained by Theorell (1934) and by Taylor & Morgan (1942) shows only one haem-linked group ionizing in the pH range 5·9–7·4, with pK about 6·6 in myoglobin and about 6·3 in oxymyoglobin and in metmyoglobin, from which it is seen that at about pH 7 the haem-linked group in metmyoglobin will be almost completely ionized. On this basis it follows that the anions F^- and CN^-, and not their conjugate acids, are bonded in metmyoglobin complexes. This conclusion has been substantiated by an independent and direct spectrophotometric study of this linked ionization in the fluoride complex, for which the value of pK was found to be 6·45 at 20° and $I = 0{\cdot}40$.

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The Mechanism of Formation of Metmyoglobin Complexes. By P. GEORGE and G. I. H. HANANIA. (*Department of Colloid Science, University of Cambridge*)

Since the formation of metmyoglobin fluoride and cyanide complexes involves the bonding of the anions but not their conjugate acids, it follows that the reaction between acidic metmyoglobin and the acid HX can be represented generally by the following set of equations in which both X^- and HX are reactants:

$$Y.Fe^+(H_2O)+X^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Y.FeX+H_2O; \quad \text{(i)}$$

$$Y.Fe^+(H_2O)+HX \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} Y.FeX+H_3O^+; \quad \text{(ii)}$$

$$\overset{+}{H}Y.Fe^+(H_2O)+X^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \overset{+}{H}Y.FeX+H_2O; \quad \text{(iii)}$$

$$\overset{+}{H}Y.Fe^+(H_2O)+HX \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} \overset{+}{H}Y.FeX+H_3O^+. \quad \text{(iv)}$$

In these equations the symbols are as defined above, and k_1, k_2, etc., are the formation velocity constants and k_{-1}, k_{-2}, etc., the corresponding dissociation velocity constants. On the assumption that the reactions of alkaline metmyoglobin, Y.FeOH, with X^- and HX are negligible in comparison with reactions (i)–(iv), the net velocity constants for the formation and dissociation of the complex, k_f and k_d, will be given by

$$k_f=\frac{H}{(K_r+H)\,(K_{HX}+H)\,(K_{Fe}+H)} \times\{K_r(k_1K_{HX}+k'_1H)+H(k_2K_{HX}+k'_2H)\}, \quad \text{(v)}$$

$$k_d=\frac{1}{(K_p+H)}\{K_p(k_{-1}+k'_{-1}H)+H(k_{-2}+k'_{-2}H)\}, \quad \text{(vi)}$$

where K_r and K_p are, respectively, the ionization constants of the haem-linked group $\overset{+}{H}Y$ in the reactant, metmyoglobin, and in the product, the complex; K_{HX} is the ionization constant of the acid HX, and K_{Fe} is the ionization constant of the iron-bound H_2O molecule in acidic metmyoglobin. The various constants are also related amongst themselves by equations of the form

$$\frac{k_1k_{-2}}{k_{-1}k_2}=\frac{k'_1k'_{-2}}{k'_{-1}k'_2}=\frac{K_p}{K_r}, \quad \frac{k_1k'_{-1}}{k_{-1}k'_1}=\frac{k_2k'_{-2}}{k_{-2}k'_2}=\frac{1}{K_{HX}}. \quad \text{(vii)}$$

The overall equilibrium constant for complex formation, evaluated in terms of total concentrations of reactants and products, will therefore be given by

$$K_{obs.}=\frac{k_f}{k_d}=\frac{K_r.(K_p+H).H.K_{HX}}{(K_r+H).K_p.(K_{Fe}+H)\,(K_{HX}+H)}\cdot\frac{k_1}{k_{-1}}. \quad \text{(viii)}$$

Kinetic and equilibrium studies of the reactions of metmyoglobin with fluoride and cyanide have been carried out over a range of pH, ionic strength and temperature. The results were found to be consistent with this mechanism, thereby confirming the assumptions made regarding the non-reactivity of alkaline metmyoglobin and the bonding of anions in the complexes, and on this basis the various rate constants and activation energies as well as the corresponding values of $\Delta H°$, $\Delta G°$ and $\Delta S°$ have been obtained.

It is interesting to note that whereas the formation of the metmyoglobin-cyanide complex by reaction of the CN^- ion is a little favoured with respect to the reaction of HCN (at 25° and $I=0{\cdot}1$, $k_1=3{\cdot}7\times10^3$, $k'_1=1{\cdot}1\times10^2\,M^{-1}\,sec^{-1}$), the formation of the fluoride complex from HF is about a million times faster than from its anion F^- (at 20° and $I=0{\cdot}40$, $k_1=1{\cdot}6\times10^{-2}$, $k'_1=1{\cdot}4\times10^4\,M^{-1}\,sec^{-1}$). The very high solvation energy of the fluoride ion may in part be responsible for this striking result.

'Hyocholic Acid', a Trihydroxy Bile Acid from Pig Bile. By G. A. D. HASLEWOOD. (*Guy's Hospital Medical School, London, E.C.* 1)

During an investigation into the use of paper chromatography for the preliminary examination of crude bile salts, Haslewood & Sjövall (1954) found that pig bile contained a substance running on paper rather more slowly than glycocholic acid. At the time J. Sjövall suggested that this might be a glyco-3:6:12-trihydroxy-cholanic acid. The bile acid probably responsible for the effect has now been isolated by chromatography, guided by paper chromatography, on Al_2O_3 of ethylated pig bile acids. The new bile acid, named 'hyocholic acid', is a trihydroxy acid, $C_{24}H_{40}O_5$ isomeric with cholic acid. It had m.p. 188–189° and $[\alpha]_D$ about $+5{\cdot}5°$ (in ethanol). Evidence so far available suggests the possibility that the hyocholic acid molecule carries hydroxyl groups at positions 3, 6 and 11 in a cholane nucleus.

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Conjugated Bile Acids of Some Primate Biles. By H. S. WIGGINS. (*Postgraduate Medical School, London, W.* 12)

Until recently the taurine-glycine ratio of the conjugated bile acids could only be estimated by the sulphur content of the purified bile salts. The quantitative estimation of all the major bile acid constituents of bile is now possible by counter current distribution (Ahrens & Craig, 1952), followed by chromatographic separation of the bile acid methyl esters, identifying the fractions by their infrared spectra (Wootton, 1953).

The ethanol-soluble fraction of gall-bladder bile was first distributed between 15 tubes in *n*-heptane/97·5 % acetic acid. The first four tubes contained the bile salts. These purified bile salts were then distributed between 30 tubes in the system, 70 % *n*-heptane/butanol (30 %)/acetic acid (10 %). Tubes 1–6 contained the taurine conjugates and tubes 12–30 the glycine conjugates. Each fraction was then hydrolysed, esterified and chromatographed.

Three specimens of human bile were analysed to obtain some idea of the variation of the taurine-glycine ratio in a single species. Bile salts of *Pan satyrus* (chimpanzee), *Cercopithecus aethiops tantalus* (tantalus monkey), *Cercopithecus mona* (Mona monkey) and two specimens of *Cercopithecus nictitans martinii* (Martin's white-nosed monkey) were also examined. The taurine-glycine ratio in the human species ranged from 1 : 1·4 to 1 : 10 and in the monkeys from 1 : 0·8 to 1 : 11·5.

The percentage composition of the acids in the taurine and glycine fractions of a single specimen of bile were the same within the limits of experimental error, except in the case of one of the Martin's white-nosed monkeys. In the human bile, chenodeoxycholic acid (3α:7α-dihydroxycholanic acid), deoxycholic acid (3α:12α-dihydroxycholanic acid) and cholic acid (3α:7α:12α-trihydroxycholanic acid) were found, the chenodeoxycholic acid predominating. These same acids were found also in the monkey bile, cholic acid being the major constituent in every case except the tantalus monkey, where deoxycholic acid was the most abundant. Lithocholic acid (3α-hydroxycholanic acid) was found in small amount in *Cercopithecus mona*, and fractions from the tantalus monkey and *Cercopithecus nictitans martinii* gave spectra in the fingerprint region similar to that of 3α:12α-dihydroxy-7-ketocholanate. The specimens were, however, too small and too impure for a positive identification to be made.

The author wishes to thank Prof. G. A. D. Haslewood, who kindly supplied the monkey bile specimens.

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Infrared Spectra of a Series of Bile Acids. By I. D. P. WOOTTON. (*Postgraduate Medical School, London, W.* 12)

During work on the identification of bile acids by infrared spectrometry, the spectra have been recorded of a number of acids possessing the same steroid nucleus but with markedly different side-chains. The spectra are all closely similar, especially in the region 1200–900 cm^{-1} (the 'fingerprint' region). This introduces difficulties in using the fingerprint as a means of identification, but, on the other hand, makes it possible that the nuclear structure of an unknown compound may be suggested by its spectrum.

Rapid Changes in Cerebral Creatine Phosphate. By P. J. HEALD. (*Biochemical Laboratories, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

Application of electrical impulses to slices of the cerebral cortex of rats or guinea pigs suspended in suitable salines, results in decreased levels of creatine phosphate in the slices; 1·5 μmoles/g. wet wt. reduced to 0·6 μmoles/g. wet wt. (McIlwain & Gore, 1951). Similar changes occur *in vivo* (Klein & Olsen, 1947; Dawson & Richter, 1950).

By means of an apparatus permitting the rapid removal of slices from the saline after or during the passage of impulses (Heald, McIlwain & Nelligan, 1954) the rates of breakdown and resynthesis of creatine phosphate in the slices suspended in a phosphate-free medium buffered with tri(hydroxymethyl)aminomethane, have been followed.

On passing impulses (for apparatus see Ayres & McIlwain, 1953), creatine phosphate was broken down at a rate of 1200–1400 μmoles/g. wet wt./hr.; breakdown was complete in 5 sec. If impulses were

switched off within 7 sec. of application, creatine phosphate was resynthesized to the original level within 20 sec. at a rate of 150 μmoles/g. wet wt./hr. After passage of impulses for 20 min. some 90 sec. was required for resynthesis of creatine phosphate, which took place more rapidly towards the end of the period than at the beginning. This period was not shortened by the inclusion of 1·3 mM-KH_2PO_4 in the medium.

Treatment of slices with 0·09 M-KCl reduced the creatine phosphate level within 30–40 sec. to a level similar to that produced by electrical impulses in 5 sec. Changes in level of inorganic phosphate were opposite to changes in level of creatine phosphate but in the case of slices treated with 0·09 M-KCl the rise in inorganic phosphate did not occur until creatine phosphate levels had fallen by 80 % of the total decrease.

The rate of creatine phosphate breakdown is within the range which can be calculated from the data of Dawson & Richter (1950), i.e. 900–4600 μmoles/g./hr. for brain *in vivo*.

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A Spectrophotometric Method for the Determination of Changes in the Thiamine Molecule.

By J. C. Somogyi. (*Physiological Institute, University of Berne, Switzerland*)

An attempt has been made to develop a method by which not only the concentration of thiamine can be determined but also changes in the molecules can be registered. This is of importance in experiments in which the thiamine molecule is cleaved, for example by the action of sulphite or of anti-thiamine factors. The ultraviolet absorption spectra of thiamine and its cleavage components have therefore been investigated under different experimental conditions.

Since the absorption spectrum of thiamine depends upon the pH of the solution and the published reports are conflicting (Peters & Philpot, 1933; Wintersteiner, Williams & Ruehle, 1935; Melnick, 1939) the ultraviolet absorption spectrum of thiamine was determined over the pH range 3 to 8. Between pH 5 and 8 the spectrum is characterized by two maxima (232–234 mμ. and 265–268 mμ.) and at pH 4 and 3 by only one (246 mμ.). This change in the spectrum brought about by alteration in pH is, according to our experiments, reversible.

Thiamine was mixed with its thiazole (4-methyl-5(β-hydroxyethyl)-thiazole) and pyrimidine (2-methyl-4-aminoethylpyrimidine dihydrochloride) components in different equimolecular proportions, thus imitating the progressive cleavage of the thiamine molecule. The maximum at 265 mμ. showed a characteristic change. The less thiamine and the more thiazole-pyrimidine the test mixture contained, the greater the decrease in this maximum. A mixture of thiazole-pyrimidine alone had no second maximum, but showed a straight line.

Analogous changes can be observed in the spectrum of thiamine if the cleavage is brought about by sulphite, as described by Williams, Waterman, Keresztesy & Buchman (1935). It seems possible that these absorption curves may be evaluated quantitatively.

The decrease of the thiamine concentration in the test mixture was followed simultaneously by the thiochrome method and by determination of the ultraviolet spectra. The value of this method in the experimental study of thiamine inactivation will be briefly discussed.

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Citrate Accumulation from Glycollate by an Oxalate Decomposing Organism. By G. C. N. JAYASURIYA. (*Agricultural Research Council Unit for Microbiology, University of Sheffield*)

The isolation and some properties of an oxalate decomposing organism have already been reported (Jayasuriya, 1953). The effect of 0·001M fluoroacetate on the oxidation of various C_2 compounds and tricarboxylic acid cycle intermediates by washed suspensions of this organism has been investigated manometrically at pH 8·0 in 0·0125M triethanolamine buffer. Fluoroacetate inhibited the oxidation of these substrates to varying degrees and in some cases (glycollate, glycine, pyruvate, succinate, fumarate, malate, oxaloacetate and α-oxoglutarate) citrate accumulated. The citrate accumulation from glycollate has been investigated further.

Citrate was determined by the method of Taylor (1953) and from 10 μM of glycollate about 1 μM of citrate was produced. Citrate was identified by paper chromatography and characterized by the isolation of the quinidine derivative.

The amount of citrate formed is directly proportional to the oxygen uptake during the oxidation of glycollate. The carbon of the glycollate metabolized could be accounted for as carbon dioxide and citrate. In the presence of a fixed amount of cells the citrate production was directly proportional to the amount of glycollate added, whereas in the presence of a fixed amount of glycollate the citrate production was independent of the concentration of cells. These results suggest that the added glycollate acts as the sole carbon source for synthesis of the citrate formed.

Citrate production from glycollate is not stimulated by the addition of the C_4 dicarboxylic acids, succinic, fumaric, malic and oxaloacetic. This does not rule out the possibility that these acids may be intermediates in citrate synthesis from glycollate, since the oxygen uptake in the presence of these acids is not much greater than in their absence, suggesting that the permeability of the cell to these substances may be the limiting factor.

These results indicate that there is a net synthesis of citrate from glycollate. A likely mechanism for this synthesis would involve the condensation of a C_4 unit with a C_2 unit. Hence in this organism it is probable that a mechanism exists for the synthesis of a C_4 unit from a C_2 unit.

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The Isolation of Plant Nucleic Acid Fractions for Purine and Pyrimidine Determinations. By H. S. A. SHERRATT and A. J. THOMAS. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Methods are readily available for the isolation of nucleic acids from animal and bacterial sources. In plants the nucleic acid concentration is much lower, and there is much interfering polysaccharide and other material which prevents direct application of existing techniques. Consequently, knowledge of the normal nucleic acids in plant tissues has been largely confined to wheat embryo. In this present work, the purine and pyrimidine composition of nucleic acid fractions, obtained from bracken, clover and rye grass whole leaves and from wheat embryo, has been examined.

The nucleic acids together with protein and polysaccharide were extracted by aqueous solutions (pH 8). This large molecular material was precipitated at pH 3. Extraction of the precipitate with boiling 10% (w/v) NaCl followed by precipitation at pH 1 yielded a fraction high in P content (Williams, 1945). Pentose nucleotides and deoxypentose nucleic acid (DNA) were obtained from this fraction (Schmidt & Thannhauser, 1945; Davidson & Smellie, 1952; Sherratt & Thomas, 1953). The purine and pyrimidine pentose nucleotides, after acid *iso*butanol extraction, were determined ionophoretically (Davidson & Smellie, 1952). The incubation with KOH in the separation procedure freed the DNA and enabled subsequent quantitative extraction of the precipitated DNA by saturated NaCl, much of the contaminating protein and carbohydrate being insoluble. After dialysis and concentration the DNA was reprecipitated to give products with 2–6% P. The purine and pyrimidine composition of these preparations was determined chromatographically (Wyatt, 1951).

The results of the base analyses and the general applications of the method will be briefly discussed.

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The Effect of Nucleotoxic Stilbene Derivatives on Globin Monolayers. By D. F. CHEESMAN. (*Department of Physiology, Bedford College, University of London*)

Haddow, Harris, Kon & Roe (1948) showed that 4-aminostilbene and certain related compounds inhibited growth of the Walker rat carcinoma 256. Some of these compounds also proved to be carcinogenic. The effect in tumour cells was shown by Dr P. C. Koller to consist in chromosome fragmentation. Active compounds were derivatives of *trans*-stilbene with a basic group in a *p*- or *o*-position and with unbroken conjugation. Substitution of one or both ethylenic hydrogen atoms reduced or eliminated activity, as did other interference with the planarity of the molecule. Any substituent in the *p'*-position gave an inactive compound.

An attempt has been made to find a system which responds specifically *in vitro* to the nucleotoxic stilbene derivatives. Such a system has been found in unimolecular films of globin.

The compounds are very insoluble in water and show no marked surface activity. If, however, a mixed solution in *iso*propyl alcohol (80 % v/v) of active stilbene (0·1 mg./ml.) and globin hydrochloride (0·5 mg./ml.) is spread on a Langmuir trough, the properties of the resultant protein monolayer differ from those of a monolayer formed in the absence of the stilbene. The latter brings about an alteration in the phase-boundary potential and a lowering of the surface pressure at which the film sets to a two-dimensional gel. The force/area diagram is essentially unaffected at surface pressures below 15 dynes/cm.

The substances investigated may be divided into three groups, according to their effects on globin films spread on 0·01 M-Na_2HPO_4/KH_2PO_4 of pH 6·8:

I. Strong effect: 4-aminostilbene (4-AS), 4-dimethylaminostilbene (4-DAS), 2'-CH_3-4-DAS, 2'-Cl-4-DAS and 1-(4'-dimethylaminophenyl)-2-(1'-naphthyl)ethylene.

II. Weak effect: 2-DAS, 4-NH_2-3'-DAS, 4-dimethylamino-α-methylstilbene.

III. No significant effect: 3-DAS, 4'-CH_3-4-AS, 4'-CH_3-4-DAS, 4-bromostilbene, 4-dimethylamino-β-methylstyrene and 4-dimethylamino-1, 2-diphenylethane.

Group I consists of substances with high growth-inhibitory activity. Of group II, 4-NH_2-3'-DAS is highly active, while the other compounds are weak inhibitors. None of the compounds of group III is biologically active.

The correlation fails with compounds bearing large hydrophobic substituents, such as 2'-$(CH_3)_2$CH-4-DAS, a biologically inactive material which penetrates into the protein film.

It appears that an active molecule is adsorbed beneath the protein, being bound at at least two sites on the polypeptide network and thus exerting a tanning action. The nature of the linkages involved is not clear. Resonance theory suggests that canonical structures of the dipolar quinonoid type contribute to the 4-AS molecule. Although the calculations of Coulson & Jacobs (1949) indicate that this contribution is small, it may be conceived that interaction with polar groups on the protein will tend to stabilize such structures, so that electrovalent linkages are established at the basic group and the 4'-position.

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DEMONSTRATIONS

Isolation of Cellular Constituents Using a Combination of the Behrens and the Schneider Techniques. By G. ADJUTANTIS. (*Postgraduate Medical School, London, W.* 12)

Behrens (1938) showed that intact nuclei could be isolated from animal tissue which had been dried in the frozen state. In the present case, liver from exsanguinated rats is frozen at −20° and dried *in vacuo*. The dried tissue is powdered and homogenized in isotonic sucrose solution (+ Versene, 0·2 %, w/v). The cellular constituents, namely, nuclei, mitochondria, submicroscopic particles and cytoplasm are separated by differential centrifugation as described by Hogeboom, Schneider & Pallade (1948). The nuclei are washed with isotonic sucrose solution (+ citric acid, 0·3 %, w/v) and identified microscopically. The mitochondria are identified by staining with janus B green, and are rich in succinic dehydrogenase, as shown by the tetrazolium method described by Pearse (1952). The cytoplasm is in clear solution, pH 6·8, containing about 4 % (w/v) protein depending on the initial dilution. The proteins can be resolved into five components by electrophoresis on filter paper using a borate buffer (pH 8·6, $I = 0·18$).

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A Microcuvette for Infrared Spectrography. By PAULINE LEWIS, H. S. WIGGINS and I. D. P. WOOTTON. (*Postgraduate Medical School, London, W.* 12)

The solubility of steroids in common infrared solvents (e.g. CS_2, $CHCl_3$) are such that one must often work at a solution thickness of 1–3 mm. This introduces difficulties in making cells with amalgamated lead or copper spacers, while no completely satisfactory cement has been found. For this reason, we have made our cells from solid rock-salt blanks, $20 \times 15 \times 6$ mm., through which is drilled a 1–3 mm. diameter vertical hole. The rough hole left by the drill is polished, and tapered polythene plugs close the ends of the hole. The cell is placed in the radiation path so that the source is imaged inside the cell and brought to a focus on the entrance slit of the spectrometer. The position of the cell is critical and controlled by micrometer screws. With this cell, a spectrum can be obtained with 50 μg. of a steroid. The technique of making these cells will be demonstrated.

Direct Determination of Serum Cholesterol. By I. MACINTYRE and MARY RALSTON (introduced by E. J. KING). (*Postgraduate Medical School, London, W.* 12)

Zlatkis, Zak & Boyle's (1953) $FeCl_3$ method for the direct determination of serum cholesterol was compared with the modified Sackett extraction method (Liebermann-Burchard) as described by King (1951). A reagent containing $FeCl_3$ and H_2SO_4 is added to serum in acetic acid, without the necessity of extracting the cholesterol and precipitating the proteins. The method is simple and the colour is stable for at least 3 hr.

Reagents

Glacial acetic acid. Aldehyde-free, by refluxing over CrO_3 for 3 hr., and then distilling twice.

Ferric chloride. 10% $FeCl_3 . 6H_2O$ in redistilled glacial acetic acid.

Colour reagent. To 0·5 ml. of $FeCl_3$ solution add 7·5 ml. conc. H_2SO_4, mix, and dilute to 50 ml. with conc. H_2SO_4. This reagent is light yellow when first mixed, but becomes colourless.

Standard cholesterol. 20 mg./100 ml. in redistilled glacial acetic acid.

Method

Test. Pipette 0·1 ml. serum into 6 ml. redistilled glacial acetic acid in a 1×6 in. test tube. Add 4 ml. colour reagent slowly down the side of the tube so that two layers are formed. Mix thoroughly to obtain even heat distribution, and allow to cool. Read the red colour against a blank (0·1 ml. water, 6 ml. acetic acid and 4 ml. colour reagent) using Ilford filter 626 (max. abs. 570 mμ.).

Standard. 5 ml. redistilled glacial acetic acid, 0·1 ml. water, 1 ml. standard, treated similarly to the test with colour reagent.

Reproducibility. Ten estimations on the same sample were in the range 235–240 mg./100 ml.

Bilirubin increases the cholesterol results by about 1·4 mg. for each mg. bilirubin.

Mean results and range for 10 samples by the $FeCl_3$ and Sackett methods were 227 and 218 mg./100 ml. (382–168 and 355–165 mg./100 ml.) respectively.

Analysis of variance showed no significant difference between the results by the different methods and the residual S.D. was $\pm$ 12 mg./100 ml.

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Zlatkis, A., Zak, B. & Boyle, A. J. (1953). *J. Lab. clin. Med.* **41**, 486.

The Determination of Serum Calcium with a Flame Spectrophotometer. By I. MACINTYRE (introduced by E. J. KING). (*Postgraduate Medical School, London, W.* 12)

The determination of serum calcium by flame photometry has proved more difficult than with Na and K for several reasons, of which spectral interference by sodium, the relatively low intensity of light emitted by calcium, and its low concentration are the most important. Preliminary precipitation as oxalate eliminates sodium interference, but this procedure has little advantage over a permanganate titration.

A flame spectrophotometer has been constructed capable of accurate determination of serum calcium, a tenfold dilution with water being the only preliminary step. An air-acetylene flame is used, the required wavelength being isolated by the monochromator of a Hilger spectrophotometer.

The emission is detected with an RCA 931. A multiplier phototube maintained at 1080 v and measured with a Pye Scalamp galvanometer.

Several types of atomizer and burner, were used; the most satisfactory were those described by Baker (1954), who generously loaned these units for trial and supplied drawings. A glass cylindrical expansion chamber was included.

The calcium emission at 423 mμ. was selected for measurement, as it was found that with 0·2 mm. slit widths sodium interference was minimal. A steady deflexion of 60 mm. is produced by sera containing 10 mg./100 ml. of calcium.

Interference effects were investigated. In general they are small and may be summarized as follows: (0·25 mM-$CaCl_2$, concentrations those in the solution sprayed); Na^+, 15 mM-NaCl depressed Ca emission by an amount just exceeding the degree of spectral interference which was 3 %. The net deflexion was lessened by 1–2 %.

K^+, 2 mM-KCl produced 1 % spectral interference. No mutual interference effects were observed. $H_2PO_4^{2-}$ in concentrations of 0·1, 0·3 and 3 mM depressed the calcium emission by 3, 3 and 10 % respectively. NH_4^+, 15 mM, depressed calcium emission by 5 %. $MgSO_4$, 0·5 mM, depressed calcium emission by 2 % producing spectral interference of the same order.

The high accuracy required of serum estimations made it necessary to incorporate 14 mM-NaCl and 0·1 mM-KH_2PO_4 in the standard calcium solutions. Biological variation in these substances is then without effect.

It is thought that dilution of urine with a phosphate solution, also incorporated in appropriate standards, may allow determinations to be made on urine by direct dilution.

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Simplified Colorimetric Blood Sugar Method. By A. M. Asatoor and E. J. King. (*Postgraduate Medical School, London*)

The Harding (1933) titrimetric blood sugar method is one of the most sensitive and accurate procedures described. The reagent consists of two parts, A, which is the $CuSO_4$ solution, and B, which contains the alkali, tartrate, etc. These are mixed 1 : 1 on the day of use. Maximum sensitivity is thus obtained, and freedom from that potent source of error, precipitated cuprous oxide, which is not uncommon in alkaline copper sugar reagents.

The colorimetric modification (Haslewood & Strookman, 1939; King & Garner, 1947) has now been simplified by using excess of Cu in the Somogyi (1931) copper tungstate deproteinization, thus providing the necessary $CuSO_4$ in the protein-free filtrate, and then adding half-strength Harding's B in measured amount to samples of the filtrates. $CuSO_4$ is likewise included in the standards. It serves as a preservative, and anti-glycolytic agent for the blood (King, Pillai & Beall, 1941), as well as the oxidizing agent in the subsequent method.

Reagents

Isotonic sodium sulphate-copper sulphate. 30 g. $Na_2SO_4 . 10H_2O$ and 6 g. $CuSO_4 . 5H_2O$/l. water. 10 % *Sodium tungstate. Harding's B* (half strength): 12 g. NaK tartrate, 20 g. Na_2CO_3, 25 g. $NaHCO_3$ and 18 g. K oxalate/l. water. *Stock glucose*: 100 mg./100 ml. isotonic sodium copper sulphate solution. *Glucose standards*: 1, 1·5 and 2·5 ml. stock glucose diluted to 100 ml. with sodium copper sulphate solution (equiv. to 80, 120 and 200 mg. glucose/100 ml. blood) and *Folin-Wu phosphotungstomolybdic acid* or *Nelson's arsenomolybdic acid*; see King (1951, p. 25).

Method

Blood (0·05 ml.) is mixed with 3·9 ml. isotonic sodium copper sulphate, 0·05 ml. tungstate added, mixed and filtered or centrifuged. 2 ml. filtrate, and 2 ml. of the standards, are treated with 2 ml. of Harding's B in ¾ in. test tubes, stoppered with cotton wool, and heated in boiling water for 10 min.; cooled, and 6 ml. of phosphotungstomolybdic acid added, and 5 ml. of water. The blue colours are read after 5 min.

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Automatic Scanning of Paper Electrophoretic Results. By D. J. R. Laurence. (*Postgraduate Medical School, London, W.* 12)

A photomultiplier operated at constant anode current is used to obtain a voltage proportional to the optical density. The protein pattern, stained with 'amidoschwarz 10B', is scanned by the photocell and the distribution of optical density is plotted automatically using a recording milliammeter.

Estimation of Salicylates in Blood Plasma. By E. J. King and Valerie Pash. (*Postgraduate Medical School, London, W.* 12)

The salicylates are extracted with ethylene dichloride, and are estimated in a similar manner to the phenol liberated in phosphatase hydrolysis (cf. King, 1951).

0·2 ml. of plasma, 1 ml. N-HCl and 6 ml. of ethylene dichloride are shaken in a centrifuge tube for 5 min., and then centrifuged for 15 min. The supernatant aqueous fraction is removed by suction, and the intermediate protein layer is either gently lifted off or filtered through a small plug of cotton wool.

4 ml. of the ethylene dichloride fraction are extracted with 3 ml. water and 2 ml. 15% (w/v) sodium carbonate, and the mixture shaken vigorously. 1 ml. dilute (33%) Folin-Ciocalteu reagent is added and the aqueous portion stirred gently with a glass rod. The mixture is warmed for 10 min. at 37°. The blue colour which develops in the aqueous layer is read in a colorimeter using an Ilford 608 light filter. A 'test blank' is prepared in the same way.

Standards of 10, 20 and 40 mg. salicylic acid/100 ml. are prepared by adding 0·2 ml. of the appropriate standard to 4 ml. water, 1·8 ml. Folin-Ciocalteu reagent and finally 3 ml. 15% (w/v) Na_2CO_3 solution. The mixture is warmed for 10 min. at 37°. A 'water blank' is prepared in the same way.

Calculation:

$$\frac{\text{test} - \text{'test blank'}}{\text{standard} - \text{'water blank'}} \times \text{standard (mg./100 ml.)}.$$

REFERENCE

King, E. J. (1951). *Micro-analysis in Medical Biochemistry.* London: Churchill.

Inhibition of Enzymes by Silicic Acid. By P. R. N. Kind, E. J. King, Valerie Pash, W. Roman and E. Schmidt. (*Postgraduate Medical School, London, W.* 12)

Mavrogordato (1926) described an apparent 'mummification' of phagocytes containing silica particles in the lymph channels of the lung. Policard (1933) observed that migrating dust cells, laden with silica, appear to suffer a slowing of their movement and final immobilization, without undergoing the lysis and disintegration which is the usual fate of phagocytic cells which have ingested bacteria or other particles. This led to the idea that slowly dissolving silicic acid, from the silica particles, has both a poisoning and a preserving action on the cell protoplasm, possibly similar to the effect of water glass or to petrification.

The failure of these silica-containing cells to undergo lysis suggested that their lytic enzymes might be poisoned by the dissolved silica, and that silicic acid might also have an inhibiting effect on other enzymes. Using standard methods of enzyme estimation, and silicic acid (neutralized, dialysed, and ion-exchange resin-treated sodium metasilicate) at several concentrations, it has been found that pepsin, trypsin, erepsin and the lysis of aseptically removed and incubated tissues, are only slightly inhibited by silicic acid in concentrations up to 0·025M. Lipase and catalase are not inhibited; acid and alkaline phosphatase, acetyl choline esterase and amylase are slightly, and urease considerably inhibited.

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Colorimetric Estimation of Silica Without Removal of Phosphate and Iron. By E. J. KING, VALERIE PASH and W. WEEDON. (*Postgraduate Medical School, London, W.* 12)

Silicate and phosphate both couple with molybdate in weak acid; only silicomolybdate is reduced if the solution is first made strongly acid (Milton, 1951). Small amounts of tissue iron likewise do not interfere if sufficient phosphate is present.

Agreement between this colorimetric modification (King, 1939) and gravimetric results on normal and pneumoconiotic lungs has been good. Mean results and range for 22 samples by gravimetric and colorimetric methods were 1·37 and 1·29 % (4·2 – 0·1 % and 4·6 – 0·1 %), respectively, of silica. Analysis of variance showed no significant difference; residual S.D. was 0·24 %.

Tissue analysis. 0·05–0·5 g. (depending on silica content) of tissue and six times its weight of Na_2CO_3 in a 25–50 ml. platinum crucible is 'smoked off' and fused to a clear melt (1–2 hr.); dissolved in water by warming; cooled, and 5 ml. of 0·44 % KH_2PO_4 (1 mg. P/ml.) added; neutralized to congo red with 10 N-H_2SO_4 and 0·2 ml. in excess added. The solution is transferred to a volumetric flask and made to 250 ml. An amount of the solution containing 0·02–0·10 mg. SiO_2 is transferred to a 25 ml. volumetric flask.

Standards. 2, 5 and 10 ml. (0·02, 0·05 and 0·10 mg. SiO_2) 'weak standard' (31·4 mg. Na_2SiF_6/l.) in similar flasks, and a *blank* of water.

Water is added to about 15 ml., and 2 ml. of 5 % ammonium molybdate in N-H_2SO_4. After 10 min. 5 ml. 10 N-H_2SO_4 are added, and then 0·5 ml. of aminonaphtholsulphonic acid reducing agent. The solutions are made up to volume, mixed, and colours read after 10 min.

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King, E. J. (1939). *Biochem. J.* **33**, 944.
Milton, R. F. (1951). *Analyst*, **76**, 431.

Colorimetric Estimation of Silica in Urine. By E. J. KING, B. D. STACY and W. WEEDON. (*Postgraduate Medical School, London, W.* 12)

Some animal urines (e.g. rat) are difficult to analyse colorimetrically for silica because of very high phosphate content and, frequently, protein. The phosphate may be difficult to eliminate by precipitation with basic ferric acetate, without loss of silica; and there may be a heavy precipitate of ammonium phosphomolybdate if the phosphate is left in according to the procedure of King, Pash & Weedon (1954). By removing most of the phosphate with calcium, or the ammonia with nitrite and protein with trichloroacetic acid, these difficulties are overcome.

(*a*) *By removal of phosphate.* 0·3 ml. of rat (or other herbivore) urine high in silica, or 3 ml. of human (or carnivore) urine low in silica, are treated in a Lusteroid or plastic test tube with 10 ml. 0·5 % $CaCl_2$ and saturated $Ca(OH)_2$ until pink to phenolphthalein, and diluted with water to 15 ml. About 50 mg. carbon (Neo Spectro MII, Columbian Carbon Co., Brooklyn, 32, or Kalbaum's Carbo Activ) are added, the mixture shaken, warmed at about 70° for 15 min., cooled and filtered (Whatman 42).

Into 25 ml. volumetric flasks are pipetted 10 ml. filtrate ($\equiv$0·2 or 2 ml. urine), and 0·02, 0·05 and 0·1 mg. SiO_2 (2, 5 and 10 ml. 'weak standard', 31·4 mg. Na_2SiF_6/l.). Water is added to about 15 ml., and 2 ml. 5 % ammonium molybdate in N-H_2SO_4 and mixed. After 10 min. 5 ml. 10 N-H_2SO_4 are added, mixed, and then 0·5 ml. of aminonaphtholsulphonic reducing agent. After a further 10 min., test and standards are diluted to 25 ml., and the colours read.

(*b*) *By removal of ammonia (and urea).* 2 ml. urine are shaken with 1 ml. 5 % $NaNO_2$, 2 ml. 20 % trichloroacetic acid and 50 mg. carbon and 1 drop caprylic alcohol for 5 min., and filtered. For rat urine, 0·5 ml. filtrate, and for human, 3 or 4 ml. are placed in a 25 ml. flask, and standards as above. Water is added to 15 ml. and 2 ml. 7 % sodium molybdate in N-H_2SO_4. After 10 min. 5 ml. 10 N-H_2SO_4 are added, mixed, and 0·5 ml. reducing agent. Solutions are diluted to the mark, mixed and read after a further 10 min.

Recoveries of added silica have been good (90–100 %) by both these methods. All chemicals and reagents must be AnalaR and silica-free.

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King, E. J., Pash, Valerie & Weedon, W. (1954). *Biochem. J.*

The Colorimetric Estimation of Aluminium. By B. D. STACY (introduced by E. J. KING).
(*Postgraduate Medical School, London, W.* 12)

This estimation depends upon the measurement of the colour of aluminium hydroxyquinolate solutions in chloroform. After removal of iron by alkaline precipitation, aluminium is completely extracted from a buffered solution (pH 5) by a 1% solution of 8-hydroxyquinoline in chloroform (Gentry & Sherrington, 1946).

The transmission curve of aluminium hydroxyquinolate shows a maximum at about 395 mμ.; Chance no. 8 OVI, and Wratten no. 2 filters with a mercury lamp in a Spekker absorptiometer give a suitable wavelength range for the photometric determination of aluminium.

Method. A suitable sample (containing 40–80 μg. Al), e.g. from the acid filtrate from a gravimetric silica determination, is pipetted into a 20 ml. graduated measuring cylinder. The sample is neutralized with N-NaOH (the volume of alkali required being previously determined with a test sample) and 2 ml. excess N-NaOH added. After making to 20 ml. the mixture is shaken and filtered. 10 ml. of the filtrate is pipetted into a 100 ml. separatory funnel containing buffer (30 ml.) and 0·1N acetic acid (10 ml.). 10 ml. of 1% 8-hydroxyquinoline in chloroform is added and the mixture shaken for 5 min. The chloroform layer is run off into a test tube containing anhydrous sodium sulphate (about 1 g.). The solution after drying is read in the absorptiometer. A 'standard blank' is prepared by shaking 10 ml. of the 8-hydroxyquinoline reagent with 20 ml. of water and 30 ml. of buffer. Standards are prepared by adding 20 or 40 μg. Al in 20 ml. of water to 30 ml. of buffer and shaking with 10 ml. of the 8-hydroxyquinoline reagent.

The reading of a 'test blank' prepared from the reagents present in the Al solution is subtracted from the 'test' readings.

Buffer. 7 parts (by volume) of M-sodium acetate. 3 parts (by volume) of M-acetic acid.

8-Hydroxyquinoline solution. 1 g. of 8-hydroxyquinoline (A.R.) dissolved in 100 ml. chloroform (A.R.).

Standard. Stock standard aluminium solution: 1·681 g. of $AlNH_4(SO_4)_2.12H_2O$ (A.R.) dissolved in 100 ml. of water. This solution contains 1 mg. Al/ml. *Dilute standard*: Stock standard diluted 1 in 100 giving a solution containing 10 μg. Al/ml.

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Gentry, C. H. R. & Sherrington, L. G. (1946). *Analyst*, **71**, 432.

The Estimation of Collagen in Silicotic Tissues. By B. D. STACY (introduced by E. J. KING).
(*Postgraduate Medical School, London, W.* 12)

This estimation depends upon the determination of hydroxyproline (Neuman & Logan, 1950*a*) from which collagen may be calculated (Neuman & Logan, 1950*b*). To investigate formalin-fixed tissue, some modifications have been made. Non-collagenous protein, hitherto removed from fresh tissue by urea treatment, is precipitated by trichloroacetic acid (TCA) from an aqueous extract of the tissue after autoclaving, whereby collagen is transformed into gelatin which is soluble in TCA (Janota, 1943; Harkness, 1952).

Hydroxyproline is then determined in an acid hydrolysate of the gelatin.

Method. A sample (100 mg.) of dried ground-fixed lung is extracted with ether. Water (5 ml.) is added, and the mixture autoclaved for 6 hr. at 30 lb. Water is added to 10 ml. and, after heating for 15 min. in a water bath, the mixture is cooled, remade to 10 ml., shaken, centrifuged and filtered. To 5 ml. of filtrate are added 5 ml. of 10% TCA and the mixture is filtered after 1 hr. A sample (5 ml.) of filtrate is evaporated almost to dryness, taken up in 6N-HCl (2 ml.), and washed with acid (4 ml.) into a hard glass test tube. The tube is sealed and autoclaved for 4 hr. at 40 lb. The contents are made to 10 ml. with 6N-HCl, shaken with charcoal and filtered. A sample of this hydrolysate is neutralized with 6N-NaOH, and, if necessary, diluted further with a neutral solution of 6N-HCl and 6N-NaOH. 1 ml. of the neutralized solution (containing 5 to 15 μg. of hydroxyproline) is mixed with 0·01M-$CuSO_4$ (1 ml.), 2·5N-NaOH (1 ml.) and 6% H_2O_2 (1 ml.). The mixture is heated for 5 min. in a water bath at $80 \pm 2°$, then cooled for 5 min. in an ice bath. 3N-H_2SO_4 (4 ml.) and 2 ml. of a 5% (w/v) solution of *p*-dimethylaminobenzaldehyde in *n*-propanol are added. The reagents are thoroughly mixed, heated for 16 min. at $70 \pm 2°$, cooled in water at room temperature and the colour read in a Spekker absorptiometer using Ilford 605 yellow green filters.

1 ml. of a standard solution containing 10 μg. of hydroxyproline (prepared from a pure sample of the amino-acid) and a blank (1 ml. of water) are prepared simultaneously with the above reagents.

Conversion factor: % hydroxyproline × 7·46 = % collagen (Neuman & Logan, 1950*b*).

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Neuman, R. E. & Logan, M. A. (1950*b*). *J. biol. Chem.* **186**, 549.

Automatic Recording Scanner for Isotope-bearing Chromatograms. By J. E. S. Bradley (introduced by C. E. Dalgliesh). (*Postgraduate Medical School, London, W.* 12)

Chromatograms carrying ^{14}C-labelled compounds are drawn between two thin-window counters (one on each side of the paper) by a stepping motor and roller system. The motor speed is proportional to the counting rate. The recording paper is driven at the same speed as the chromatogram. Timing marks are made to indicate the counting rate. This system in conjunction with a ratemeter and pen recorder, overcomes most of the difficulties associated with the use of a constant scanning speed, when widely differing counting rates are encountered.

Apparatus for Simultaneously Measuring Ultraviolet Adsorption and Radio-activity in the Eluate from Chromatographic Columns. By J. E. S. Bradley (introduced by C. E. Dalgliesh). (*Postgraduate Medical School, London, W.* 12)

Ultraviolet radiation of suitable wavelength is isolated from the continuous spectrum of a hydrogen arc, using simple filters. The radiation passes through a cell, into which the effluent drips, and thence on to a photomultiplier. The optical density of the solution in the cell is recorded continuously, and correlated with successive tubes of the fraction collector. The apparatus is demonstrated in operation in the region of 275 mμ.

The radio-activity of the effluent is monitored simultaneously by flowing it over the window of a counter, and is recorded on the same paper.

A Simplified Apparatus for the Photo-synthesis of [^{14}C]Starch and Sugars. By R. W. Dutton. (*Postgraduate Medical School, London, W.* 12)

The leaf chamber consists of a 7 l. bell jar with a desiccator top as lid, the two being immersed in a larger inverted bell jar as a water bath. A uniform temperature of 30° is achieved by adjustment of the rate of outflow of the warmed water while the level is maintained by cold water from a constant level chamber.

Light is supplied by sixteen 30 W Osram 'Striplites' giving a light intensity of 2250 metric candles.

A simple gas circuit serves for the liberation of the carbon dioxide and the recovery of any residual activity as barium carbonate at the end of the run. The air is circulated continuously and can be monitored by a thin end-window Geiger counter.

This apparatus can handle 20 g. of leaf with the complete assimilation in 10 hours of 1·2 g. of carbon dioxide. It has the advantage of simple construction from standard laboratory apparatus and does not depend on the use of sunlight.

Apparatus for Maintaining and Rapidly Transferring Sliced Tissues. By P. J. Heald, H. McIlwain and D. Nelligan. (*Biochemical Laboratories, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5)

With the present apparatus, tissues can be transferred from good metabolic conditions to fixing solutions in about 0·25 sec., and exposure to agents for periods down to 1 sec. has been examined. Tissue slices of about 100–120 mg. and 3 cm.^{2} in area are held fully open and horizontal in 5 ml. saline by two grids in a plastic frame. The frame can be quickly transferred and the grids opened, releasing the slice, still extended, to a fixing reagent which penetrates it rapidly. The frame fits to a small beaker and with a rubber shield makes a closed system to which moist oxygen (or other gas) is led as a continuous stream through this saline, which is thus gassed and caused to circulate around the tissue.

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